α -DIALKYL AMINO ACID TRANSAMINASE: PURIFICATION AND MECHANISM

By

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ABBREVIATIONS

The following is a list of abbreviations of chemical compounds and enzymes which are used in the text.

AIB	α-aminoisobutyric acid
PAL	Pyridoxal
PAM	Pyridoxamine
PLP	Pyridoxal 5'-phosphate
PMP	Pyridoxamine 5'-phosphate
GOT	Glutamic-Oxaloacetic Transaminase

INTRODUCTION

Statement of the Problem

The purpose of this research was to elucidate the mechanism of the enzymatic degradation of DL-α-methyl-α-amino-*n*-butyric acid (isovaline) and related α-dialkyl amino acids. This was accomplished by 1) enrichment and isolation of an organism containing an enzyme that acted readily on these substrates; 2) purification of the enzyme responsible for the first step of the degradation; 3) partial analysis of both the reaction capabilities and the substrate specificities of this protein; and (4) interpretation of the reaction mechanism and enzyme reaction specificity in terms of related systems.

Background

The mechanisms of the most common reactions of amino acid metabolism in mammals, e.g., transamination, oxidative deamination, and certain eliminations, require cleavage of the substrate α-hydrogen. Other reactions, such as the reverse aldol cleavage of serine (1) or deamination of histidine (2) do not involve the α-hydrogen but depend upon an electron donating or withdrawing group attached to the β-carbon. Amino acids with two alkyl groups attached to the α-carbon have been found to be resistant to enzymatic attack in animals (3-6), although Weissbach *et al.* (7) noted that α-methyl analogs of 3,4-dihydroxyphenylalanine and several other aromatic amino acids were decarboxylated to the appropriate amines by a pyridoxal 5'-phosphate (PLP)-dependent aromatic amino acid decarboxylase from guinea pig kidney at about one hundredth the rate of the parent compounds. While the rate was too slow to consider this a physiologically significant reaction, the finding was mechanistically significant because it did point out directly, the non -essentiality of an α-hydrogen for PLP-catalyzed enzymatic decarboxylation.

In 1908, Ehrlich (8) reported that a racemic mixture of isovaline was resolved by yeast cultures. Twenty years later the thesis work of den Dooren de Jong (9) as reported by Stephenson (10) listed several common organisms which were able to grow with isovaline or α-amino-isobutyric acid (AIB) as their nitrogen source. They were *B. polymyxa*, *A. aerogenes*, *B. herbicola*, *E. coli.*, and *S. marcesens*. These reports have not been confirmed.

The most recent report of microbial metabolism of α-dialkyl amino acids was that of Aaslestad and Larson (11) who isolated a pseudomonad which utilized α-amino isobutyrate (AIB) for carbon. Dialyzed cell extracts of their organism catalyzed the release of carbon dioxide from this amino acid when pyruvate and PLP were added to the reaction solutions. Alanine and acetone were identified as products. In a subsequent preliminary report (12) these workers announced a partial purification and characterization of the enzymatic system. The PLP and α-keto acid requirement persisted through a ten-fold purification of the decarboxylating activity. Decarboxylation-transamination by a single enzyme was proposed.

From the evidence presented and from an understanding of enzymatic transamination reactions the following sequence can be written for the proposed reaction:

$$\text{AIB} + \text{PLP-Enzyme} \longrightarrow \text{carbon dioxide} + \text{acetone} + \text{PMP-Enzyme} \tag{1}$$

$$\text{Pyruvate} + \text{PMP-Enzyme} \longrightarrow \text{alanine} + \text{PLP-Enzyme} \tag{2}$$

The details of the overall reaction which appears to have no clear analogy in previously studied PLP-dependent enzyme systems were not reported.

A similar enzyme became the object of the research presented here. In order to understand the mechanistic implications of such a reaction sequence, it will be instructive to consider briefly, first, some of the available information about the mechanisms of PLP-catalyzed reactions.

Mechanisms of Pyridoxal Catalysis

General

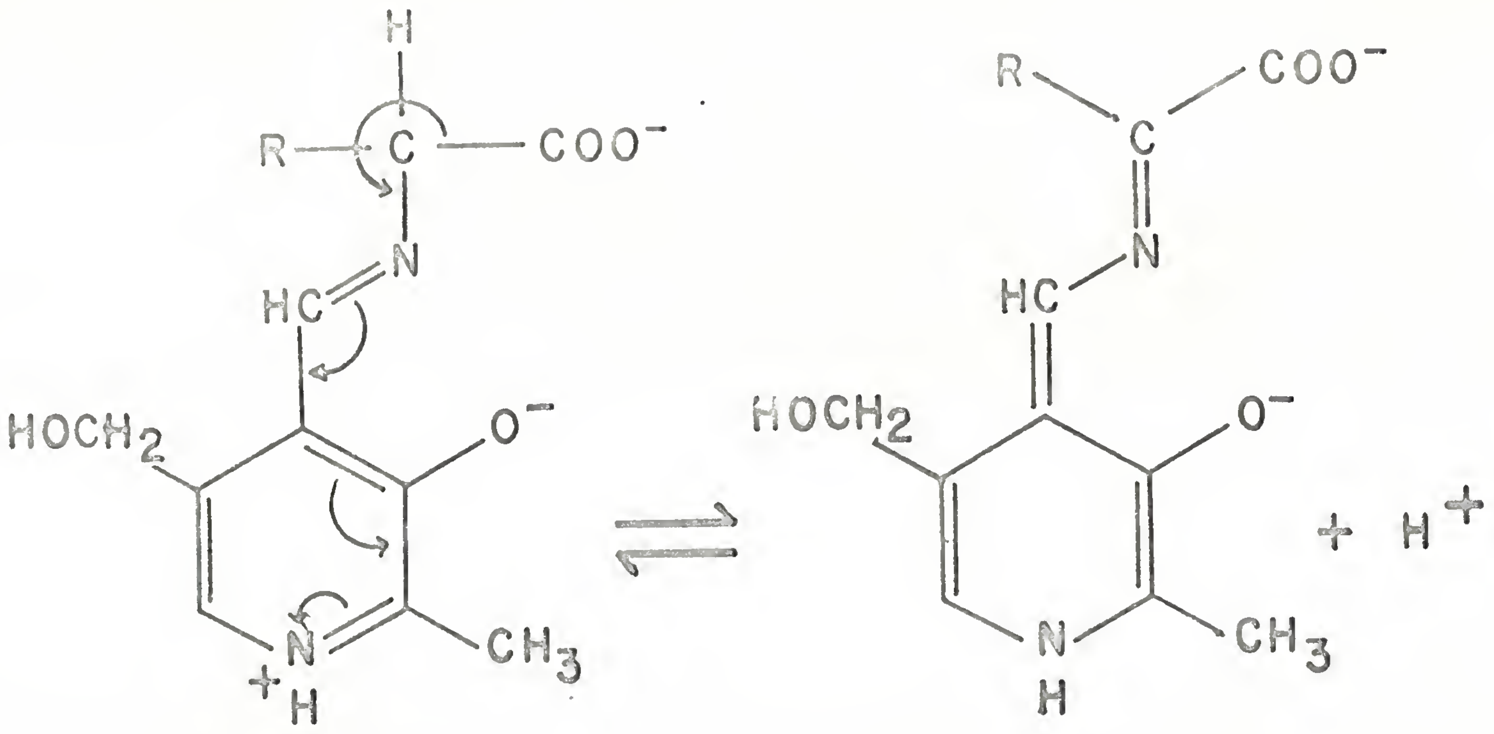
The discovery twenty years ago by Snell (13,14) that a variety of amino acids reversibly transferred their amino group to pyridoxal (PAL) during autoclaving led to his suggestion that this compound might serve as an amino group carrier in enzymatic transamination. This fact was soon established in his, and other, laboratories (15-17) and the confirmation stimulated further exploration of the non-enzymatic reactions of pyridoxal with amino acids (18-20).

Using structural analogs, the components of the substituted pyridine ring which were essential for transamination were determined (21-23). These were found to be the formyl group, the heterocyclic nitrogen *ortho* or *para* to the formyl, as well as a free hydroxyl *ortho*, or possibly *para*, to the formyl function (24). With 4-nitro-salicylaldehyde it was found that the nitro group could replace the protonated pyridine nitrogen, showing that the electron withdrawing capacity of the substituted ring was an important property which assisted transamination.

These several findings served as the experimental foundation for the well-known proposals of the general mechanisms of pyridoxal catalysis published in 1954 by the workers in Snell's laboratory (21) and independently by Braunstein and Shemyakin (25). These mechanisms have been reviewed and extended considerably since their original formulation (26-31) but the basic concepts have remained valid.

The initial step in all such reactions is believed to be the formation of an aldimine Schiff base between the amino acid and the formyl of pyridoxal (I, Figure 1). The other bonds to the amino acid α-carbon are weakened in this complex by the inductive effect of the electronegative pyridine nitrogen felt through the conjugated system which extends to the amino nitrogen. In a subsequent step one of these bonds about the α-carbon is cleaved to form a ketimine intermediate (II, Figure 1). This ketimine may be hydrolyzed, leading to transamination, or may return to the aldimine form prior to hydrolysis.

Thus two critical steps occur in any PAL-catalyzed reaction mechanism which determine the ultimate products. They are first, labilization of a particular bond about the amino acid α-carbon in the aldimine to form the ketimine and second, protonation of the ketimine either at the formyl carbon, leading to hydrolysis at the α-carbon and production of carbonyl products and the amine form of the catalyst, or protonation at the α-carbon followed by hydrolysis of the resulting aldimine to yield amine products and the aldehyde form of the catalyst.



I

II

Figure 1. Proposed structures of the aldimine (I) and ketimine (II) complexes of pyridoxal with an amino acid. Cleavage of any one of the bonds about the α-carbon of the amino acid may occur in the conversion of the aldimine to the ketimine as indicated by the circular arrow including all three bonds. Loss of the α-hydrogen is illustrated here.

Decarboxylation

PAL-catalyzed, non-enzymatic decarboxylation of α-hydrogen bearing amino acids is not easily demonstrated because this reaction proceeds much more slowly than the competing reactions which are initiated by removal of the α-hydrogen atom (28). In order to avoid the complication of these competing reactions Kalyankar and Snell (20) studied non-enzymatic decarboxylation by PAL with the α-dialkyl amino acid, AIB. Not only did the expected PAL-catalyzed decarboxylation of this amino acid to isopropylamine and carbon dioxide occur, but, a decarboxylation-transamination was observed in which pyridoxal was converted to pyridoxamine (PAM), and the amino acid carbon skeleton to acetone and carbon dioxide. They suggested the reaction schemes of Figure 2 to explain their findings. The sequence on the right including intermediate III is the now accepted mechanism of PAL-catalyzed straight α-decarboxylation initially proposed by Snell's group (32,33) and by Westheimer (34,35), in which the α-carboxyl is cleaved directly to form the ketimine. This mechanism, unlike that suggested by Braunstein and Shemyakin (25) and Werle and Koch (36), would not involve the α-hydrogen of a normally configured amino acid. The sequence to the left, including intermediate IV, is the proposed mechanism of decarboxylation-transamination. The formation of the ketimine is the same in both reactions; the difference is the position of protonation of the

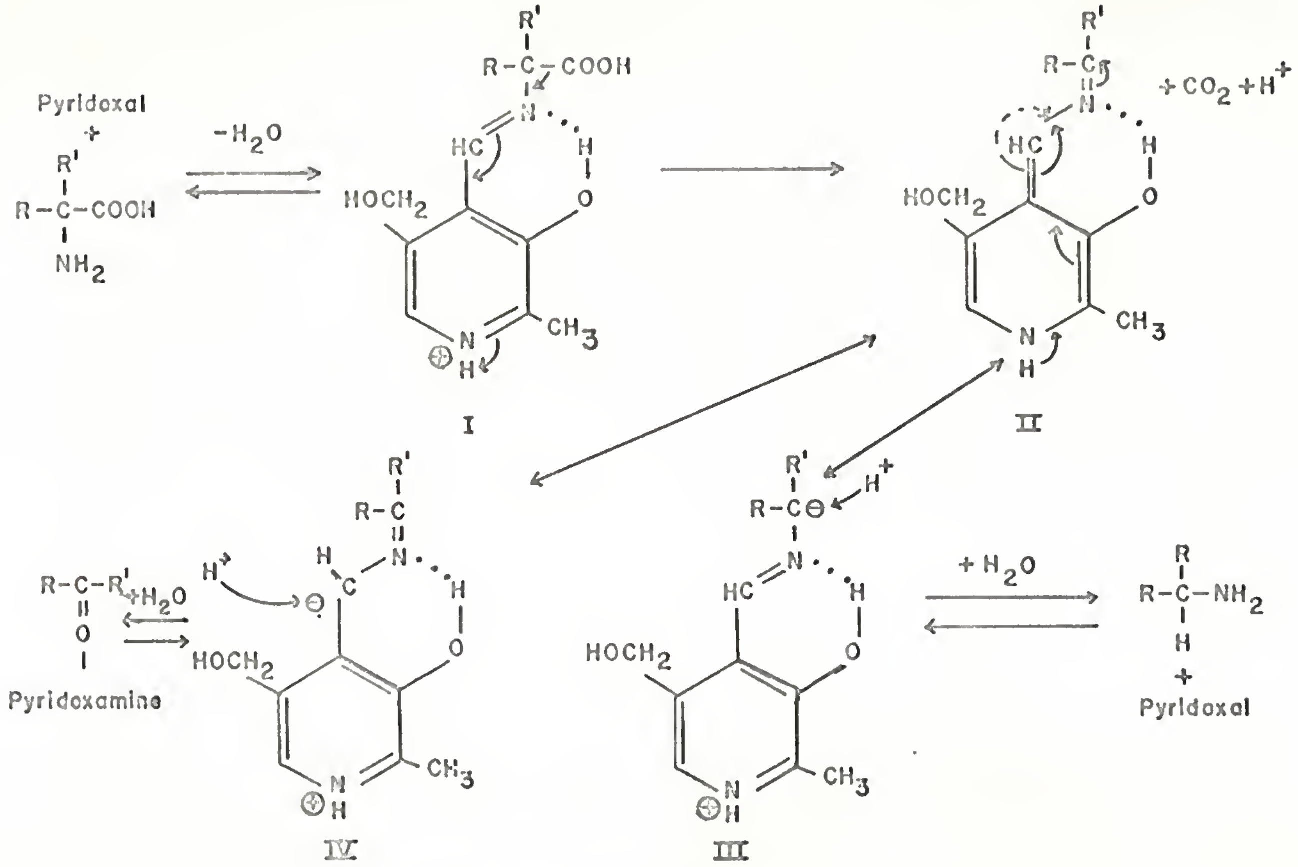


Figure 2. PROPOSED COURSES OF NON-ENZYMATIC PYRIDOXAL-CATALYZED DECARBOXYLATION AND DECARBOXYLATION - TRANSAMINATION (SNELL) (20).

ketimine intermediate and consequently the site of hydrolysis which breaks the complex. Equation (1) of the reaction proposed by Aaslestad and Larson (11) is the enzymatic analogy for this non-enzymatic decarboxylation-transamination.

The non-involvement of the α-hydrogen in enzymatic decarboxylation of natural amino acids was first indicated when it was determined that deuterium was not lost from α-deutero tyrosine during decarboxylation with tyrosine decarboxylase (35). Further, Belleau and Burba (37) prepared both stereo isomers of α-deutero tyramine by decarboxylation of α-deutero tyrosine and unlabeled tyrosine by tyrosine decarboxylase in water and deuterium oxide, respectively. They then compared the rates of oxidation of these enzymatically prepared substrates by a stereo -specific amine oxidase with the rates of oxidation by the same enzyme of chemically synthesized R and S α-deutero tyramine. The results proved that decarboxylation occurred with retention of configuration (i.e. the proton acquired from the solvent was added at the configurational position occupied by the α-carboxyl before cleavage to form the ketimine).

Transamination

Snell's proposed mechanism (28) of one half reaction of enzymatic transamination is shown in Figure 3. The formation of the initial aldimine (II) is represented as

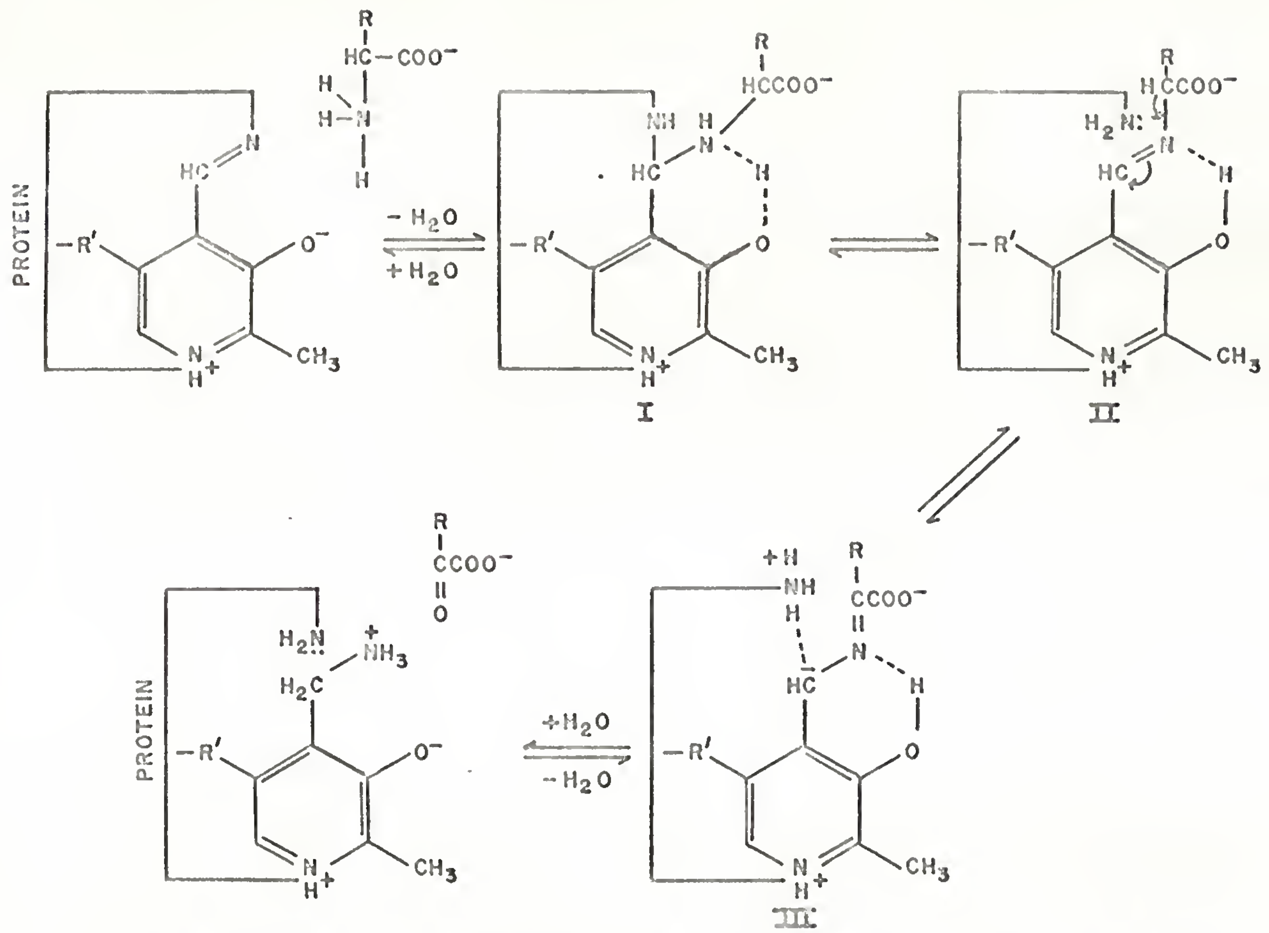


Figure 3. PROPOSED MECHANISM FOR ONE HALF REACTION OF ENZYMATIC TRANSAMINATION (SNELL) (28).

a transaldimination, since it is currently believed that the aldehyde function of PLP is bound as an aldimine to a lysyl ε-amino group of an enzyme when not complexed with substrate (38,39,28). Formation of the ketimine (III) in this reaction occurs by cleavage of the α-hydrogen.

The essence of the transamination reaction is that protonation of the ketimine may occur at either the formyl carbon or the α-carbon so that hydrolysis leads to transfer of the amino group to or from the co-factor. The second half reaction of transamination, with the appropriate α-keto acid substrate, is merely the reverse of the half reaction as written.

It is now well established that the only transamination mechanisms consistent with abundance of data gathered (40-45) are ones in which the PLP form of the enzyme is converted to the PMP form by the amino acid substrate followed by regeneration of the PLP-enzyme by the α-keto acid substrate. Some of the most convincing data for this conclusion include the separate isolation of PLP and PMP forms of glutamic-oxaloacetic transaminase (GOT) after reaction with the appropriate substrate (41), and the finding that the GOT apoenzyme is activated by either form of the co-enzyme (42).

Elaborate kinetic studies have been performed by Velick and Vavra (43), and Henson and Cleland (44) with GOT, and by Hopper and Segal (46) with glutamic-pyruvic transaminase. Again the results were consistent only with a shuttle mechanism in which the substrates interact one at a time with the two different forms of the enzyme. In addition, substrate inhibition studies by some of these workers (43,44) provided strong evidence that all substrates occupy the same binding site sequentially.

Both half reactions of transamination are reversible (40) and measurements of exchange have been made with homologous amino-α-keto acid pairs in which one member of each pair was initially radioactive (47,48).

All transaminases studied thus far are specific for only one amino acid stereomer, normally the L form (30), although a D-amino acid specific transaminase has been purified from Bacillus subtilis (49). Reversibility studies (48) verified that the product amino acid of transamination has the same configuration about the α-carbon as the substrate amino acid. This may be looked upon as retention of configuration in the overall reaction, analogous with enzymatic α-decarboxylation.

Recent spectral and optical rotatory dispersion measurements of the interaction of α-methylaspartate with GOT (50) have led to the proposal of a detailed mechanism of the reversible half reaction of enzymatic transamination which is a refinement of, but in complete agreement with, the essential steps of the earlier proposed mechanism.

Reaction Specificity of PLP Catalysis

Non-enzymatic systems

As might be expected, in the non-enzymatic reactions of PAL with amino acids, more than one reaction may occur simultaneously leading to a variety of products (18,19). The structure of the particular amino acid involved and the reaction conditions, such as pH, ionic strength, or inclusion of certain polyvalent metal ions determine which bonds about the α-carbon become cleaved.

For example, careful accounting of all species at the end of the reaction of α-methylserine with PAL in the presence of Cu^{++} (20) revealed that the amino acid underwent either decarboxylation-transamination which involved cleavage of the α-carboxyl, or cleavage of the α-methylol group. In addition, the alanine produced by the latter reaction underwent subsequent transamination which required labilization of its α-hydrogen.

Enzymatic systems

With the aid of the non-enzymatic model systems, as much or more has been deduced about the detailed reaction mechanisms of vitamin B_6 catalysis than those of any co-enzyme. However, the understanding of the steps of these mechanisms does not explain, and in fact, seems to make more obvious the absence of an explanation for the reaction specificity and greatly enhanced rates displayed in the analogous enzymatic reactions.

The emerging understanding of protein structure and concepts of macromolecular flexibility, and their influence upon binding and catalysis at the active site (51,52,45), is providing models to explain these phenomena. Changes in the conformation of the enzyme protein, induced by groups on the approaching substrate molecule may be involved in many enzyme reactions (51) to aid binding and orientation for catalysis. The presence of bulky substituents on the substrate, which apparently are not directly involved in the reaction mechanism is often required in order for binding or catalysis to be observed (51). Also, the fact that enzymes are specific for one enantiomer of an optically active amino acid pair and may not be inhibited by the non-substrate member (53,54), illustrates the importance of the configuration of groups about one atom to binding as well as reaction.

Observations of the sensitive relationship between total substrate structure and configuration, and enzyme specificity are manifold. The recent work on the specificity of glutamine synthetsase (55) and the studies with citrate cleavage enzyme (56) are excellent cases in point. The exploration by Jenkins et al. (40) of heat stabilization and inhibition of GOT by substrate analogs demonstrated the importance of both substrate carboxyl groups to binding by this PLP enzyme.

Recently, Dunathan (57) proposed a model to explain the reaction specificity of PLP enzymes. It drew in part upon the conclusions made earlier by Perault et al. (27) from extensive quantum mechanical calculations with the molecular orbital method. These earlier workers argued that all the bond labilizations observed to occur at the α-carbon of an amino acid-PLP aldimine complex were energetically favorable because of the gain in delocalization energy acquired by extention of the conjugated system as a result of the bond cleavage.

Dunathan emphasized first that, if this gain in resonance energy was to aid kinetically in the bond-breaking process, the sigma bond to be broken must have a geometry in the transition state which allows overlap with the pi -electron cloud of the conjugated PLP aldimine. Maximum sigma-pi overlap would occur when the bond to be cleaved was oriented perpendicular to the plane of the pyridine ring. The validity of this argument was well supported by results of reaction rate studies with similar chemical systems which were sterically restricted from such overlap (58-60). This overlap is possible since the nature of the hybrid bonding in the aldimine complex confines all the atoms of the conjugated system and the α-carbon of the amino acid to the same plane (see Figure 17).

Second, Dunathan made the reasonable assumption that in the aldimine the binding of the amino acid α-carboxylate anion in the enzyme active site controlled the orientation of the bonds about the substrate α-carbon, relative to the plane of the pi-electron cloud of the co-factor. By variation of the position, relative to the plane of the conjugated system, of this carboxylate binding site on different enzymes, the proper bond about the α-carbon of each enzyme's substrates could be specifically oriented in a position for cleavage. Thus, the specificity of protein binding of the α-carboxyl group dictated the reaction specificity of the enzyme with regard to the first critical step, bond cleavage to form the ketimine. Presumably, additional factors controlled the site of protonation of the ketimine and further steps.

This model is surely an oversimplified explanation of the factors which are involved in the control of the reaction specificity of these enzymes, particularly in that it neglects the obvious importance of the other valences of the α-carbon in binding (40). Nevertheless, it is hard to deny the validity of the argument that certain geometries relative to the plane of the pyridine ring will have a lower activation energy for bond cleavage and that there is a reasonably restricted and specific site on the protein for α-carboxylate binding which will influence the orientation of the α-carbon bonds relative to the ring plane. The predictions of this model appear to fit the published data on the reaction specificities of individual PLP enzymes.

The reaction mechanism of the α-dialkyl amino acid transaminase which was the subject of this study exhibited properties both of a decarboxylase and a transaminase. For this reason it was considered useful to investigate the details of the mechanism of this enzyme. The reaction specificity which was revealed appeared inconsistent with the predictions of the model proposed by Dunathan. The effect of this apparent inconsistency on the validity and restrictions of his speculations will be discussed after the mechanism for the enzyme and some of its properties have been described.

MATERIALS AND METHODS

Materials

Chemicals

All commercial chemicals except isovaline were reagent grade or better. Lower grade commercial isovaline, which was determined to be greater than 90 per cent pure by titration, showed only a single ninhydrin sensitive spot on paper and thin layer chromatograms. This material was used in growth media but was crystallized from aqueous acetone (61) for enzyme assays.

PLP was purchased either from The California Corporation for Biochemical Research or Sigma Chemical Co. PMP was purchased from The California Corporation for Biochemical Research.

Identity and radio purity of the commercial isotopes used were checked by chromatography and, when necessary, for organic solvent extractible or volatile, acidic impurities. Radioactivity measurements were made in 10 ml Bray's scintillation fluid (62), primarily with a Packard Tricarb liquid scintillation spectrometer (Model 3003).

Chemical syntheses

One-^{14}C- and 2-^{14}C-DL-isovaline were synthesized from butanone, ammonia and potassium cyanide by a Strecker reaction (63) following the protocol of Levene and Steiger (61) with slight modifications. $K^{14}CN$ was used for synthesis of 1-^{14}C-DL-isovaline, whereas 2-^{14}C-butanone prepared from 1-^{14}C-acetate was used for synthesis of 2-^{14}C-DL-isovaline.

The separate reactions required for the synthesis of 2-^{14}C-DL-isovaline are outlined in Figure 4. One-^{14}C-acetyl chloride was prepared in 75 per cent yield by addition of a six-fold excess of freshly distilled benzoyl chloride to 60 mmoles of dry sodium acetate (64) containing 2.0 mc 1-^{14}C-acetate. The volatile product distilled as it was formed and was collected in a receiving vessel which was submerged in an acetone-dry ice bath and vented through a calcium chloride containing drying tube. When the initial exothermic reaction subsided, the mixture was held at 100° until distillation ceased. The ^{14}C-acetyl chloride was stored, sealed at -20°.

Freshly prepared ethylmagnesium bromide was converted to the less reactive diethylcadmium, for ethyl addition to the acetyl chloride, by reacting dry cadmium chloride with the Grignard reagent. One hundred mmoles finely powdered cadmium chloride was added slowly with stirring to 180 mmoles (based on the assumption of complete reaction of the initial magnesium content) of iced ethylmagnesium bromide. The resulting ethereal slurry was refluxed for

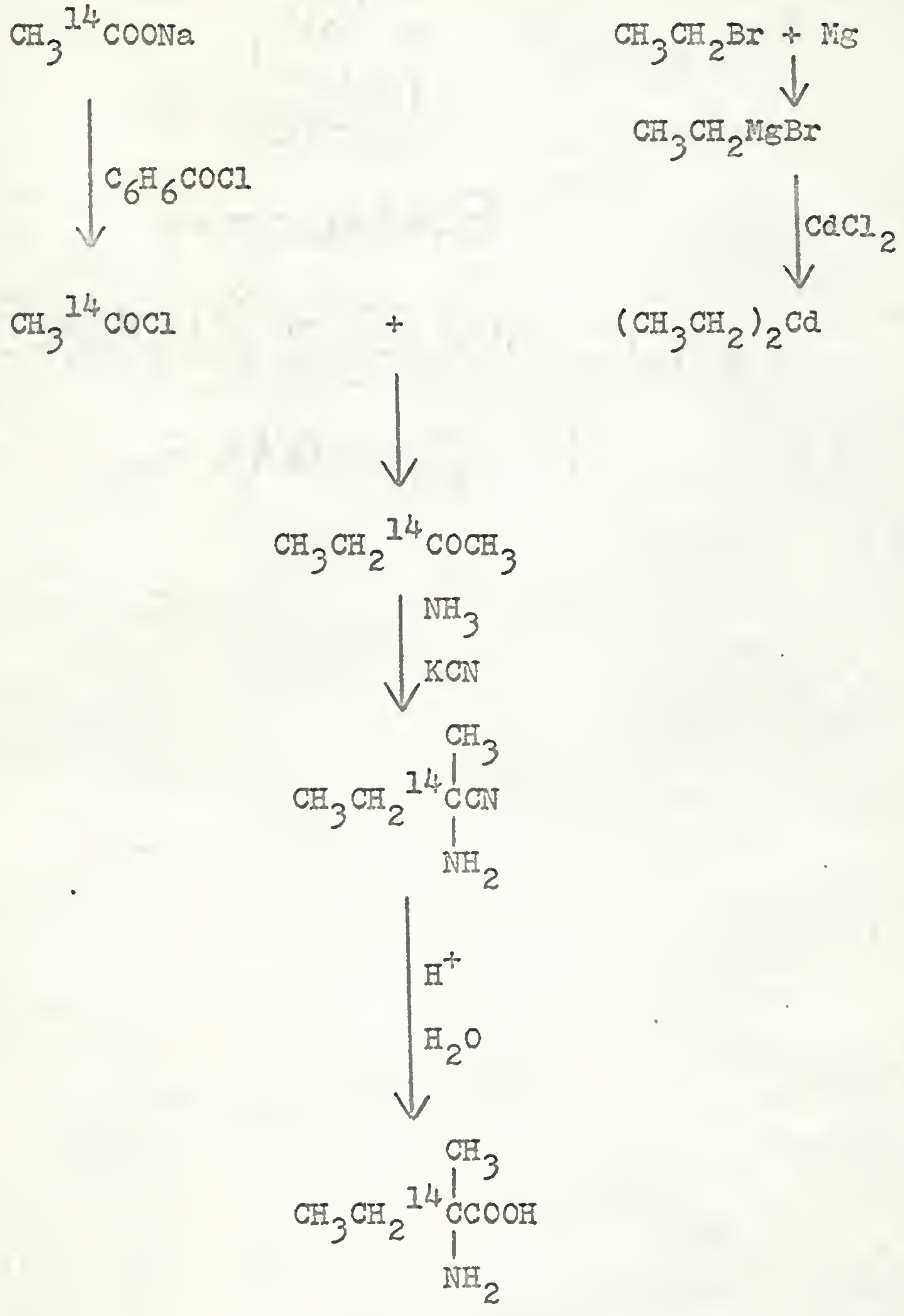


Figure 4. Reaction sequence followed in the synthesis of 2-^{14}C-DL-isovaline.

90 minutes with stirring. Ether was then removed under reduced pressure while 90 ml xylene was being added to replace the solvent.

The previously prepared 1-^{14}C-acetyl chloride was brought to 60 mmoles total with unlabelled, freshly distilled acetyl chloride, diluted with 15 ml xylene and added slowly with stirring to the organometallic compound. The bath temperature was maintained at 0° throughout the addition, and then the reaction was refluxed for 60 minutes. The addition product was hydrolyzed by adding, sequentially, 15 g ice and 40 ml 6 M sulfuric acid.

The xylene phase was saved and the aqueous phase was saturated with sodium chloride and extracted twice with 75 ml fresh xylene. The combined xylene extracts were washed twice with 20 ml of 5 per cent sodium carbonate and finally with 20 ml water. After drying over magnesium sulfate this organic phase was submitted to fractional distillation.

The fraction between 50 and 78° (butanone, bp = 79.6°) (65) contained 50 per cent of the radioactivity initially present in the washed xylene. A 2,4-dinitrophenylhydrozone prepared from a small amount of this material, melted between 116-118° (butanone 2,4-dinitrophenylhydrazone, mp = 117°) (65) and was radioactive. The ^{14}C-butanone was used in the Strecker reaction without further purification. The yield was 30 per cent based on ^{14}C content.

A portion of the radioactive butanone (approximately 0.5 mc) was diluted to 40 mmoles with freshly distilled, unlabelled butanone for the Strecker reaction which was accomplished as by Levene and Steiger (61) through isolation of the amino acid hydrochloride.

This product was dissolved in 50 ml of water, placed on a 4 cm diameter column containing 450 g washed IRC-120 (H^+) and washed free of chloride with deionized water. Isovaline was eluted with 2 N ammonium hydroxide and the volatile solvent was removed under reduced pressure. The residue was taken up in water, decolorized by filtration through charcoal, and the amino acid crystallized by addition of acetone (61). The crystals were dried to constant weight at 110° and the specific activity determined by liquid scintillation spectrometry. The yield was 60 per cent based on butanone.

The infrared spectrum was taken in Nujol with a Perkin -Elmer Infracord and compared with that of crystalized, commercial isovaline. Chromatograms of the product on paper and silica gel-G thin layers were identical to those of the commercial amino acid. Confirmation of the identity of these compounds was obtained during analysis of the products of enzymatic degradation (below).

Methods

Bacterial cultures

All cultures contained the salts of Rickenberg, Yanofsky and Bonner (66) as modified by Dempsey (67) but excluding the nitrogen source, ammonium sulfate. This salt solution, hereafter described as RYB salts was supplemented with appropriate carbon and nitrogen sources as dictated by the needs of each experiment. Stocks of the organisms isolated for this study were maintained on agar slants of RYB salts containing 0.2 per cent DL-isovaline or 0.2 per cent DL-isovaline plus 0.2 per cent glucose. Liquid cultures of one liter or less were aerated by shaking. Absorbancy at 650 mµ was used to measure cell growth. Unless otherwise indicated, cultures were incubated at 30°.

For large scale cultures one liter of freshly grown bacteria was used to innoculate each carboy containing 16 liters of medium. Aeration was with sterile air introduced through a fritted glass sparger. Foaming was suppressed with General Electric Antifoam 60. The cells were harvested in a Sharples centrifuge then suspended in 0.01 M potassium phosphate, pH 7.5, for lyophilization. Dried cells were stored at -20° until used for enzyme purification.

Enzyme assays

During enzyme purification and for a number of experiments with purified enzyme, assay involved measurement of the acetone produced by decarboxylation-transamination of AIB with pyruvate. The neutral ketone product was separated from unreacted pyruvate by extraction from alkalinized reaction solutions into carbon tetrachloride. Acetone 2,4-dinitrophenylhydrazone was prepared in this solvent by a modification of the procedure of Greenberg and Lester (68) and the absorbancy of the soluble derivative determined at 420 mμ. Acetone production was linear during the first 10 per cent of the reaction and increased linearly with protein concentration under the conditions employed.

The assay contained 20 mM sodium pyruvate, 20 mM AIB, 50 mM potassium phosphate, pH 7.5, plus 0.1 mM PLP added from an acidified, aqueous stock solution just prior to assay. When the PLP concentration in the reaction solution was critical a fresh solution was prepared. Reaction was initiated with up to 0.05 ml enzyme and carried out at 30° with either boiled or no enzyme in controls.

Up to 0.5 ml of the reaction mixture was pipetted directly into 0.2 ml 5 N sodium hydroxide which had been previously placed in an 18 X 150 mm screw capped culture tube. Acetone was extracted from this basic aqueous phase with 10 ml carbon tetrachloride on a rotary shaker for 15 minutes. The aqueous phase was discarded and 3.0 ml 0.2 per cent 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid added. The tubes were shaken another 20 minutes to allow formation and extraction of the neutral acetone

2,4-dinitrophenylhydrazone. The aqueous phase was again discarded and the organic phase washed twice with 0.5 N sodium hydroxide. The absorbancy of the 2,4-dinitrophenylhydrazone in carbon tetrachloride could be measured directly in the reaction tube with a Bausch and Lomb Spectronic 20 colorimeter. An identically treated sample from the control was used as a blank. For more accurate measurements, absorbancy was determined with a Beckman DU spectrophotometer.

A standard curve was constructed by following the same isolation procedure with aliquots of the assay solution containing known amounts of acetone. Absorbancy was converted to µmoles acetone from the standard curve. One unit of activity was defined as one µmole acetone produced per minute, and specific activity was units per mg protein. Protein was determined by the method of Lowry, et al. (69) using a bovine serum albumin standard.

Butanone formation from decarboxylation-transamination of isovaline could be measured similarly, however, a simpler quantitation of this ketone was possible using 2-^{14}C-DL-isovaline as amino acid substrate. The assay procedure was the same as used to measure acetone production through the termination of reaction with base. Ten ml toluene was added at this point instead of carbon tetrachloride and the ketone extracted into this solvent (using some of the 2-^{14}C-butanone prepared for the synthesis of 2-^{14}C-DL-isovaline, it was determined that 15 minutes' shaking with 10.0 ml toluene quantitatively extracted the butanone from 0.5 ml or less of the alkalinized reaction solution). An aliquot of the toluene layer was counted in Bray's solution to quantitate the butanone. Negligible quenching was noted when up to 5 ml of the toluene phase was counted.

For direct measurement of decarboxylation, the ^{14}C-carbon dioxide released from 1-^{14}C-amino acid substrates was trapped in hyamine hydroxide and counted by liquid scintillation spectrometry. A technique similar to that devised by Cuppy and Crevasse (70) was employed, using serum stoppered scintillation vials as the reaction, and carbon dioxide collection, vessels. The assay solution in a volume of 0.8 ml was added and the vial sealed. Two tenths ml of enzyme appropriately diluted with buffer was injected from a syringe to start the reaction. Reaction was halted and carbon dioxide released by injecting 0.2 ml 2 M sulfuric acid. After carbon dioxide capture, the hyamine-containing centerwell was wiped dry and placed directly in Bray's scintillation fluid for ^{14}C-carbon dioxide determination. This solvent was slightly less efficient than the toluene based scintillation fluid normally used to measure radioactivity in hyamine hydroxide. However, because it is more tolerant of water, less variability of counting rate due to aqueous condensation which accumulated inside the centerwells during reaction and carbon dioxide collection was observed with Bray's fluid. All counts were corrected for quenching with ^{14}C-toluene internal standard and were compared to the background values of non-enzyme controls.

Exchange between alanine and pyruvate was measured by ether extraction of the 1-^{14}C-pyruvate formed from 1-^{14}C-L-alanine. At the end of incubation up to 0.5 ml of a reaction solution similar to that described above but containing 1-^{14}C-alanine and unlabelled pyruvate was pipetted into screw capped tubes containing 0.2 ml 2 M sulfuric acid. Fifteen ml anhydrous ether was added and the aqueous phase extracted for 30 minutes on a rotary shaker. Aliquots of the ether layer were counted in 10 ml of Bray's solution. The efficiency of the pyruvate extraction procedure was determined with reaction solutions to which known amounts of 1-^{14}C-pyruvate had been added. An approximation of the initial reaction rate could be obtained if extraction was accomplished before more than a few per cent of the radioactivity had been converted to pyruvate.

The less-ether-soluble 1-^{14}C-α-keto butyrate, formed by transamination of 1-^{14}C-DL-α-amino-n-butyrate was separated from the labelled amino acid substrate on a Dowex 50 (H^+) column. In these assays enzymatic reaction was stopped with heat and 1.0 ml of the reaction mixture placed directly on top of a 1 X 12 cm column. The acids were washed through with 10 ml of water and 0.1 ml of the eluate was counted to measure the radioactive α-keto acid formed.

To measure transamination of non-radioactive amino acids, 1-^{14}C-pyruvate was used as the α-keto acid substrate, and the 1-^{14}C-alanine produced was separated from the labelled substrate on a similar column. After the unreacted ^{14}C-pyruvate was washed from the column the bound amino acids were driven off with 2 N ammonium hydroxide and 0.1 ml of this eluate analyzed for radioactivity. Corrections were made for the quenching caused by the acid or base present in the column eluates.

Enzyme purification

Sephadex G-200 was prepared for columns in cold 0.02 M potassium phosphate, 0.05 M potassium chloride, pH 7.5. Depending on sample size either a 25 X 100 cm laboratory column (Pharmacia Fine Chemicals, Piscataway, New Jersey), or a 4.5 X 85 cm plexiglas column constructed locally, was used for enzyme purification. After a column was poured it was equilibrated with the same buffer containing 50 μM PLP. Earlier columns were eluted by descending buffer flow; however, more successful fractionations were achieved later using pre-sized beads of 40-60 μ diameter and ascending elution.

Removal of salts, such as ammonium sulfate, from protein solutions, and buffer changes, were accomplished on short columns of Sephadex G-75, equilibrated with the appropriate buffer. The columns were packed with a volume of gel calculated to have an excluded volume equal to 120 per cent of the sample volume.

Ion exchange chromatography of proteins was accomplished with Schleicher and Schuell Type 40 DEAE-cellulose. The resin was washed as described by Peterson and Sober (71) prior to equilibration with buffer.

Various size DEAE-cellulose columns were prepared depending on the amount of protein to be fractionated. Generally, a ratio of 2.5 mg protein per ml of packed column was sought. Most successful enzyme elutions were made with a linear, potassium chloride gradient from 0.05 M to 0.5 M in 0.02 M potassium phosphate buffer, pH 7.5.

Similar procedures with DEAE-Sephadex, A-50 resulted in smearing of the eluted enzyme peak so that this medium was discarded in favor of cellulose.

Chromatographic analyses

Ascending paper chromatography of amino acids was accomplished on Whatman No. 1 filter paper using a lutidine:collidine:water:diethylamine solvent (72).

Thin layer chromatography of amino acids was on glass plates coated with silica gel G (Research Specialties Co., Richmond, California) using phenol:water, containing sodium cyanide (73). Amino acids were revealed with ninhydrin.

Butanone 2,4-dinitrophenylhydrazone was chromatographed on the same support. The solvent systems used are pointed out with the experimental results.

Radioactivity of non-quenching substances on thin layer plates was accurately quantitated by scraping 1 X 3 cm sections (approximately 50 mg) of dried unsprayed gel from the plates into a scintillation vial and suspending this powder in 10.0 ml of a 4 per cent suspension of thixotropic gel powder (Cab-o-sil, Godfrey L. Cabot, Inc.). There was virtually no quenching encountered with this system.

Washed Dowex 50 W- X 4 (H^+) was used for amino acid and α-keto acid separations on 1 X 12 cm columns.

RESULTS

Selection and Growth of Organisms

Enrichment on isovaline as sole carbon source

Soil from an area previously found to yield successful enrichments on isovaline had been kept in a bottle of tap water to which fresh DL-isovaline was added periodically. The first enrichment in this project was carried out with this suspension. The soil inoculum was added to RYB salts containing 0.1 per cent ammonium sulfate and 0.4 per cent DL-isovaline. Growth was visible after 2 days and an aliquot was then transferred to fresh liquid medium and shaken for 4 days. Samples of this well-grown culture were diluted in sterile saline and streaked on plates containing the same medium in 2 per cent agar. Small white colonies became visible after about 4 days at 30° but reached full size only after 10 days at the same temperature. Growth was the same at room temperature and even slower at 37°. A single colony was selected and restreaked on agar. An apparently pure strain of a gram negative organism was isolated by several such transfers and was then maintained on agar slants of the same medium. Microscopic observations revealed long, (6-7μ) actively motile rods. This bacterium grew with a doubling time of 4.5 hours on both isovaline and AIB. All attempts to increase the growth rate on the α-dialkyl amino acid substrates by such means as variation of substrate concentration, pH, temperature, trace metal or vitamin content were unsuccessful. Only when the cells were grown on a rich peptone-sugar medium could the doubling time be shortened to 2 hours. Such a medium was unsuitable for this project since presumably it would lead to catabolite repression of the α-dialkyl amino acid degrading enzymes.

Experiments designed to detect the nature of the enzymatic attack by this organism on the α-dialkyl amino acid were unsuccessful. When 1-^{14}C-DL-isovaline was used in the growth medium, up to 90 per cent of the labelled carboxyl group was trapped in hyamine suspended over liquid cultures grown in sealed flasks. This revealed that it was indeed isovaline which was being metabolized and also that both enantiomers were utilized. However, no ^{14}C-carbon dioxide release could be detected when toluenized cell suspensions or extracts prepared either with a French Pressure Cell, by sonication, or by alumina grinding were incubated with the radioactive substrate. Addition of PLP and pyruvate (or several other α-keto acids) which were required for the decarboxylation-transamination reaction (11) did not produce any detectable alteration of the substrate. Various co-factors and metals, added either alone or in elaborate mixtures, were also ineffective. Thin layer chromatograms of the incubated reaction mixtures suggested that no reaction had occurred since the only radioactive spot present coincided with that of the substrate.

When cells were shaken with isovaline at a concentration which would lead to one-half full growth in liquid culture and the remainder of the carbon requirement was provided in the form of any one of a number of compounds suspected as intermediates in the degradation of isovaline, growth reached only the extent possible with the amount of isovaline present.

Isolation of an organism utilizing α-dialkyl amino acids for nitrogen

The failure to determine the pathway by which DL-isovaline was degraded by this bacterium stimulated further search for a less resistant biological system. A number of microorganisms available locally were screened for their ability to do any of the following:

1) utilize isovaline as carbon source for growth;
2) utilize this amino acid for nitrogen in a medium containing glucose but devoid of any other nitrogen supply;
3) release ^{14}C-carbon dioxide from 1-^{14}C-DL-isovaline when allowed to grow in Difco Pen Assay medium (Difco Laboratories, Detroit, Michigan) plus 1-^{14}C-DL-isovaline.

B. herbicola was not available for this survey, however, the findings of den Dooren de Jong (8) were not confirmed with E. coli, A. aerogenes S. marcesens or B. polymxa. S. cerevesiae did not degrade isovaline although yeast had been reported to do so (7).

A single organism was discovered which both utilized isovaline for nitrogen and released the labelled carboxyl as carbon dioxide but was unable to grow on isovaline for carbon. None of the other bacteria tested showed activity of any sort.

The active organism, which had been initially isolated as a contaminant of stock cultures of E. coli B obtained from Dr. A. L. Koch, was suggested to be in the genus Pseudomonas by the following findings:

a) short, motile, gram negative rods when grown with isovaline + glucose and RYB salts;

b) aerobic growth only and alkaline reaction, on mannitol, maltose, lactose, Simmons citrate, and Russell double sugar agar and in malonate broth (all products of Difco Laboratories, Detroit, Michigan);

c) urea not hydrolyzed.

This organism, designated Ps-1, was ultimately used as the source of enzyme for this project. When cells of Ps-1 fully grown in glucose + isovaline were sedimented and resuspended in either 0.4 per cent DL-isovaline or 0.4 per cent AIB plus RYB salts, no significant growth occurred even after a week of shaking. The doubling time during logarithmic growth in the glucose + isovaline medium was approximately 2 hours. Thus, Ps-1, was distinct from the first isovaline utilizing organism isolated in this laboratory and also from that studied by Aaslestad and Larson (11) which grew on AIB as sole carbon source.

The nature of the enzymatic attack by Ps-1 on α-dialkyl amino acids

The observation that Ps-1 was able to acquire the amino group of isovaline for nitrogen metabolism and also released the α-carboxyl as carbon dioxide was consistent with a decarboxylation-transamination like that proposed by Aaslestad and Larson (11). Because pyruvate and PLP stimulated release of carbon dioxide in the latter system, these compounds were tested for their effect on the ability of cell free extracts of Ps-1 to decarboxylate isovaline (Table 1). The results supported such a mechanism.

Enzyme induction

Preliminary to attempts to purify the α-dialkyl amino acid transaminase from Ps-1, conditions were sought which would induce maximum synthesis of the enzyme.

Variation of the specific activity in crude extracts of cells grown out in 0.2 per cent DL-isovaline + 0.2 per cent glucose over the pH range from 6.0 to 7.5 was slight

TABLE 1

^{14}C-Carbon Dioxide Release from 1-^{14}C-DL-Isovaline by Cell Free Extracts of Ps-1[a]

Reaction[b]	Additions		CPM X 10^{-2}
	Pyruvate	PLP	
1	+	+	122
2	+	-	77
3	-	-	6

a) Lyophilized cells (0.4 g) were suspended in 16.0 ml 0.06 *M* potassium phosphate, pH 7.5 and sonicated at 10° for 15 minutes. The 43,000 X *g* supernatant of this sonicate was assayed.

b) Reaction 1 contained 60 umole 1-^{14}C-DL-isovaline (20,000 cpm), 60 umole sodium pyruvate, 0.4 umole PLP, 180 umole potassium phosphate, pH 7.5, and 1.0 ml cell extract in a final volume of 3.0 ml. Reactions 2 and 3 were identical to 1 with the exclusion of the indicated compounds. Incubation was for 5 hours. Radioactivity values were compared to those of acidified enzyme containing controls. The ^{14}C-carbon dioxide assay is described in *Methods*.

as was the variation when the initial concentration of DL-isovaline ranged from 0.1 to 0.4 per cent.

The activity produced in cells grown with DL-α-amino-*n*-butyrate was 50 per cent of that present in isovaline grown cells. When ammonia was available, alone or with isovaline, less than 2 per cent was present. Thus, the enzyme was both inducible and subject to catabolite repression.

Two tenths per cent isovaline + 0.4 per cent glucose were chosen as substrates for large scale production of bacteria.

Large scale cultures for enzyme production

Two 100 liter cultures of Ps-1 were prepared during the course of purification studies. In the first, the medium contained RYB salts plus 0.2 per cent DL-isovaline and 0.4 per cent glucose. pH was initially set at 6.0 because of a slight indication from preliminary studies of greater enzyme synthesis at this pH. Harvest was accomplished at 36 hours when the growth rate decreased sharply. The second 100 liter culture contained, in addition to RYB salts, 0.15 per cent DL-isovaline plus 0.15 per cent AIB for nitrogen. The higher total nitrogen as well as 0.5 per cent glucose was used to increase the total cell yield in this volume of medium. The initial pH was also raised to 7.5 to preclude cessation of growth due to acid production in the higher glucose concentration, and also because the hoped for higher enzyme yield had not been realized at the lower pH in the first batch culture. Cells again were harvested at 36 hours. The yield, after lyophilization, from the first culture was 90 g and, from the second, 141 g dry cells.

The presumption of a higher, total enzyme yield in the second culture was, unfortunately, short lived since extracts of these cells had only one-half the specific activity found in extracts prepared from the first culture.

It would be unwise to form conjectures about the reasons for the difference in specific activity in these two cultures considering the grossness of control over conditions in such large cultures. In view of the preliminary studies of the effect of pH and isovaline concentration on induction, one would tend to eliminate these factors as causes in themselves. The higher glucose concentration in the second medium may have been sufficient to make more pronounced, existing catabolite repression. Introduction of the second nitrogen source, AIB, in the second instance was the most obvious change and may be related to the cause of the reduced induction. Certainly, in retrospect, a more extensive study of the effects of carbon source and concentration, as well as of nitrogen source, on enzyme synthesis, should have been conducted before large scale production was attempted.

Enzyme Purification

There is no published purification of an α-dialkyl amino acid transaminase. In the verbal presentation of their preliminary report (12), Aaslestad and Larson discussed steps which had allowed a ten-fold purification of the enzyme studied in their laboratory. They mentioned sensitivity of the activity in crude extracts to dialysis and inhibition of activity by sodium ions. The affect of dialysis probably arose from loss of co-factor since activity could be partially recovered by subsequent addition of PLP. They reported an apparent K_{PLP} of 40 μM.

The inhibition by sodium ion could be relieved by overwhelming concentrations of potassium ion. In fact, they found that the enzyme was protected both during dialysis and heat treatment by reasonable high (0.5-1.0 M) potassium chloride concentrations.

Although there appeared to be some differences between their system and that of Ps-1 there seemed little reason to doubt that the reaction catalyzed was essentially the same. For example, in this laboratory it was found that as soon as the enzyme was removed from the environment of the cell extract, no activity was measurable without including PLP in the assay. The apparent K_{PLP} found, using the crude extract was approximately 5 μM. As a precaution against denaturation, 50 μM PLP was included whenever feasible during the purification, although it was found later that the purified enzyme remained reasonably stable when stored for long periods of time in the absence of added co-factor.

A detailed study of the effect of various salts on activity was not conducted; however, certain incidental observations were made. The activity was not changed when concentrations of potassium chloride as high as 1.0 M were included in protein solutions. Activity was not lower when the sodium rather than potassium salt of pyruvate was used in the assay, but was diminished 65 per cent when the assay was conducted in mM sodium pyrophophate buffer. It was not determined whether the additional sodium ion or pyrophosphate ion was responsible.

To maintain ionic strength during the various purification steps and to decrease the possibility of inhibition by sodium ion, an 0.05 M potassium chloride, 0.02 M potassium phosphate buffer at pH 7.5 was used during the enzyme purification, except where pointed out. For brevity this system will be designated KCl-phos or KCl-phos + PLP when 50 µM PLP is also present. Unless noted otherwise, all protein solutions were maintained between 0 to 12° during purification and stored at either -20 or +5°.

Initial purification studies

A number of standard procedures which have been useful in batch enzyme preparations were tested on a small scale with the hope of finding one which would allow at least a two-fold purification from the supernatant of 43,000 X g centrifuged sonicate without great loss of total enzyme activity. This supernatant, adjusted to pH 7.5 and containing approximately 25 mg protein per ml, was used for the preliminary studies.

Procedures which were unsuccessful will be mentioned only briefly, for reference during possible future purifications.

Neutral, aqueous 4 per cent solutions of either protamine sulfate or dihydrostreptomycin sulfate, added to the crude supernatant in increasing amounts raised the A_{280}/A_{260} ratio measured after the precipitate was removed, however, the treatment caused loss of up to one-half the enzyme activity, which was not recoverable from the precipitate.

Variable results were obtained with isoelectric precipitation. Activity began to come out of solution at about pH 5.2 and over 60 per cent could be precipitated, and later recovered, by lowering the pH to 4.9. However, below this pH a large amount of the total protein commenced to precipitate. It was noted for the first time, during the pH studies, that no activity could be detected in the dissolved precipitates unless PLP was added to the assay. Generally, there was a 25 to 30 per cent loss of total activity as a result of acid treatment. It appeared that this method of purification, which sometimes gave about two-fold purification over the crude supernatant, might be refined sufficiently to become useful, but the results of these small scale experiments were too variable to risk loss of a large proportion of the enzyme in a scaled up experiment.

Potassium chloride (0.5 M), but no combination of substrates, protected the enzyme in the crude extract, from heat up to 53°. However, little total protein was precipitated after 30 minutes at this temperature.

Final enzyme purification

A summary of the most successful purification is shown in Table 2. Specific activity and yield values refer to that portion which was carried to further purification. The final preparation from this purification was used for purity determinations and studies of the reaction except where noted. Cells from the second batch culture were used in the purification.

Preparation of cell free extracts.---Lyophilized cells were prepared for sonication by suspension in KCl-phos + PLP at a concentration of 50 mg per ml. Twenty-five ml volumes of the suspension were sonicated with the large probe of an MSE 60 watt ultrasonic oscillator. The suspension, in a jacketed glass cell, was cooled by circulating ice water. It was determined that 15 minutes'

TABLE 2

Purification of α-Dialkyl Amino Acid Transaminase[a]

Purification Step	Volume ml	Protein mg	Specific Activity $\frac{\text{uM acetone}}{\text{min mg}}$	Yield %
1. Cell sonicate	1400	36,400	0.058	100
2. 10^5 X g supernatant	1350	14,300	0.14	94
3. 33-50% $(NH_4)_2SO_4$	70	3,000	0.38	56
4. Sephadex G-200	180	967	1.04	47
5. 1st DEAE-cellulose	150	215	4.5	46
6. 2nd DEAE-cellulose	10.7	23.9	8.2	10
7. 50% $(NH_4)_2SO_4$	2.6	13.5	10	6

a) From 60 g dry cells. Details of the procedure are described in the text.

sonication was sufficient to release a maximum amount of Lowry reactive material into the 43,000 X g supernatant. All enzyme activity remained in the supernatant after this centrifugation. More than 90 per cent of the activity remained in the supernatant after centrifugation of the whole sonicate at 100,000 X g for 90 minutes, and this provided a greater increase in specific activity than the lower speed centrifugation. The 100,000 X g supernatant, divided into working size volumes, was stored at -20° and served as starting material for all further purification steps. The activity was stable for several months in this form.

Ammonium sulfate fractionation.---Ammonium sulfate precipitation was used to concentrate protein solutions for passage over gel filtration columns and also to effect a two-and one-half-fold purification from the 100,000 X g supernatant. A saturated ammonium sulfate solution was prepared at room temperature, with ammonium hydroxide added, until fifty-fold diluted sample measured pH 7.5. The concentrated solution was added slowly, with stirring, into the protein solution which also had been adjusted to pH 7.5. When the protein solution was 33 per cent saturated addition was stopped, but stirring continued for 15 minutes. The precipitate was then isolated by centrifugation and saturation raised to 50 per cent which caused a much heavier precipitate to form. This was isolated as above and both precipitates were dissolved in a minimum volume of KCl-phos + PLP.

Assay of the dissolved 33 per cent ammonium sulfate precipitate usually revealed less than 10 to 15 per cent of the recovered enzyme activity, whereas 85 to 90 per cent was found in the suspended 33 to 50 per cent ammonium sulfate precipitate. Invariably, however, up to 40 per cent of the total, original activity was lost. In addition, the total remaining activity calculated by assaying the 33 per cent saturated supernatant prior to saturation to 50 per cent was less than the value calculated after assay of the dissolved 33 to 50 per cent precipitate. The concentration of ammonium sulfate actually present during the assay of the 33 per cent supernatant was calculated to be 14 m*M*. Possibly this salt concentration produced some inhibition during the assay. Presumably the ammonium sulfate concentration in the assay solution of the dissolved 33 to 50 per cent precipitate was negligible. Aaslestad and Larson (12) had remarked that their enzyme was uninhibited by ammonium ion; however, it is not known if they used the sulfate salt. Thus it appears that there is an at least partially reversible, detrimental effect upon the enzyme of ammonium sulfate.

Gel filtration.---To determine if the enzyme could be resolved with Sephadex gels a portion of the 100,000 X *g* supernatant was mixed with slurries of G-25, 50, 75, 150 and 200 which had been equilibrated with KCl-phos. After stirring for 2 hours to allow equilibration of the enzyme with all available volume, the gels were allowed to settle and an aliquot of the supernatant assayed. The activity was lower only in the sample containing G-200, indicating access of the enzyme to the pore volume of this gel only.

The dissolved 33 to 50 per cent ammonium sulfate precipitate which contained 3 g protein in 70 ml was cleared of insoluble material by centrifugation and the density increased with sucrose. This sample was layered on the top of the 4.5 cm diameter column (see Methods) and eluted with KCl-phos + PLP at a flow rate of 20 ml per hour. The elution pattern of an early, smaller scale run on the 2.5 cm diameter column is shown in Figure 5, and is typical of elution patterns from both size columns.

As can be seen in this figure, the enzyme activity was well resolved from the bulk of large molecular weight material eluting in the excluded volume. The transaminase had an elution volume to excluded volume ratio of 1.7. This ratio was used by Andrews (74) with a number of standard proteins to develop a means of molecular weight estimation by gel filtration. Comparison of the ratio, 1.7, with data accumulated in that study allows a molecular weight estimation of about 150,000 for the α-dialkyl amino acid degrading enzyme.

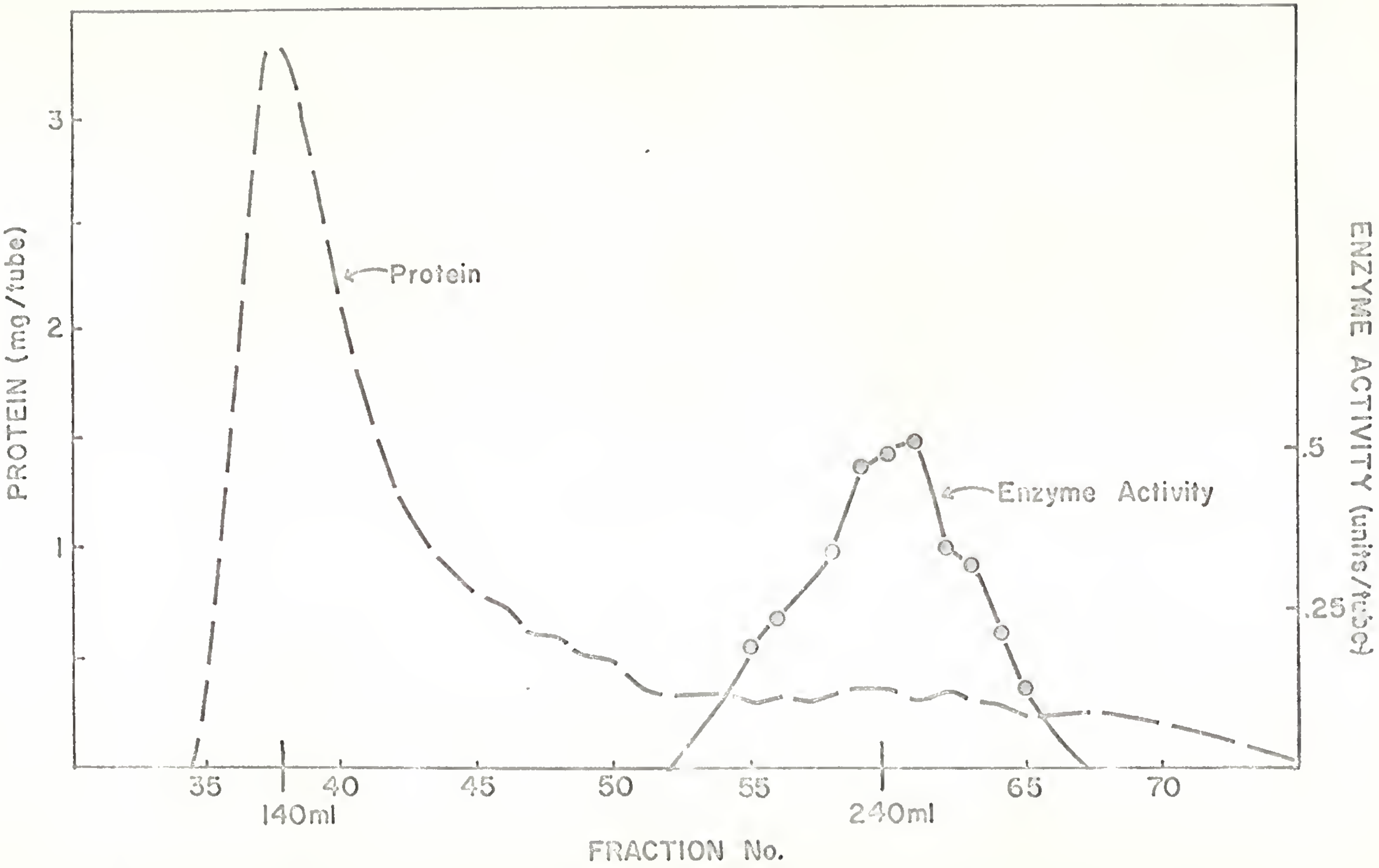


Figure 5. Gel filtration through Sephadex G-200 of the dissolved 33 to 50 per cent ammonium sulfate precipitate. Approximately 25 mg protein in 2.5 ml buffer plus 2 per cent sucrose was layered on top of a 2.5 X 100 cm column and eluted in 4.5 ml fractions at a flow rate of 20 ml/hour.

The most active fractions from this column were about three-fold purer than the ammonium sulfate fraction. The treatment proved to be very gentle since recovery totalled essentially 100 per cent of the activity placed on the column.

Ion exchange with DEAE-cellulose.---The most active fractions from the gel filtration step contained 967 mg protein in 180 ml. These were combined and added directly to a 2.5 X 100 cm column packed to 85 cm with DEAE-cellulose which had been equilibrated with KCl-phos, pH 7.5. The protein solution was allowed to soak into the cellulose bed and was washed with 450 ml of the equilibration buffer to remove any poorly bound protein. When protein was no longer detected in the effluent, the enzyme was eluted with a linear potassium chloride gradient starting with 1,000 ml in each reservoir (Figure 6). All the enzyme activity recovered from this column appeared in a single sharp peak near the center of the major protein peak. Since 95 per cent of the added activity was recovered from the column, the exclusion of stabilizing PLP from the buffer system during elution appeared not to be permanently detrimental to the enzyme. The free PLP which was present in the initial protein sample was detected by its yellow color and fluorescence under ultraviolet light and this compound eluted in a peak following, but slightly overlapping the enzyme peak. Although discrete protein bands were not

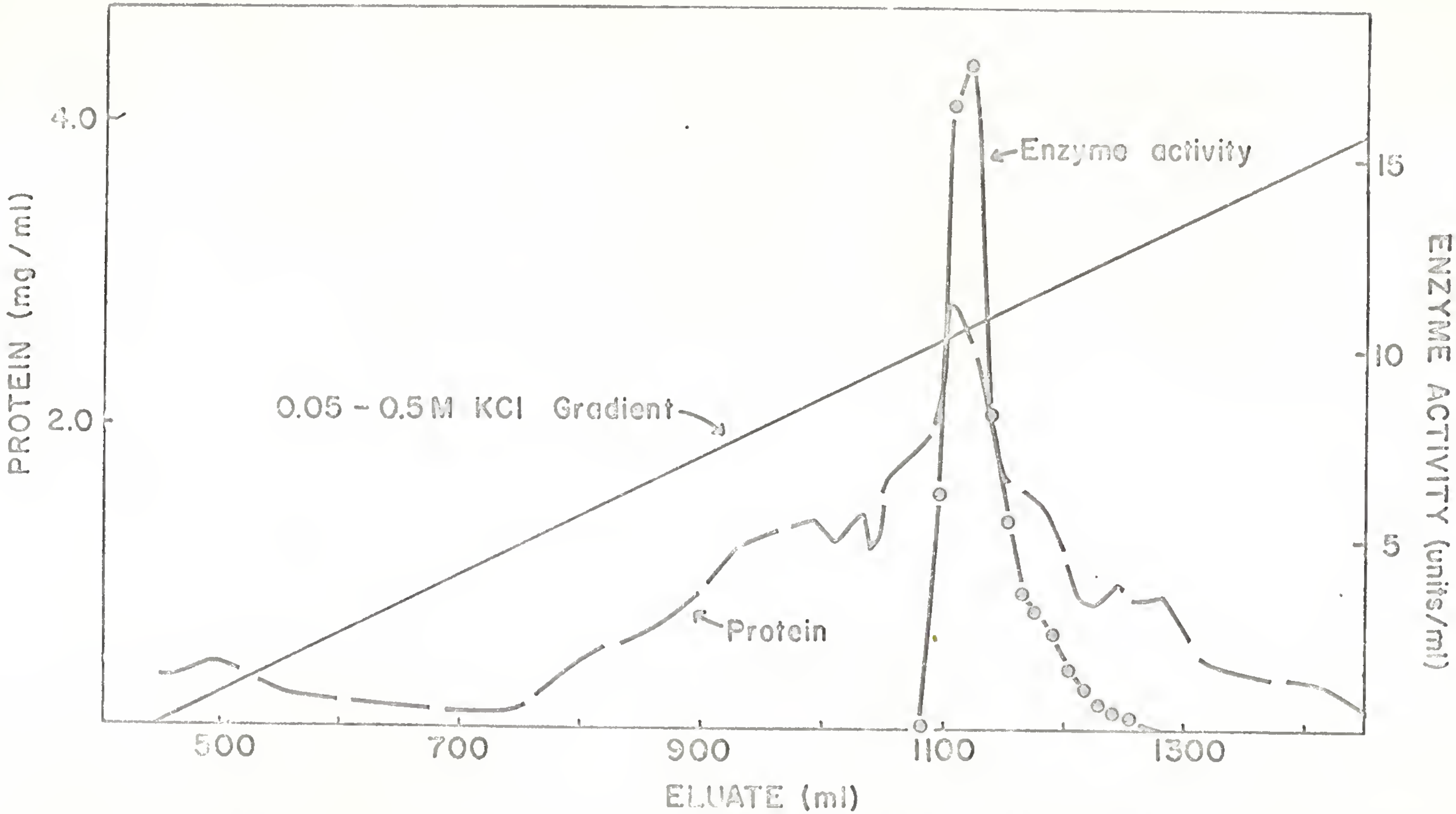


Figure 6. Gradient elution from DEAE-cellulose of the most active fractions from the gel filtration step outlined in Table 2. Fractions of approximately 20 ml were collected at a flow rate of 50 ml per hour. Other details of the procedure are presented in the text and Step 5 of Table 2.

separated on the column, the most active fractions were purified four-and one-half-fold over the last step.

Cleaner separation from contaminating proteins was obtained by a second fractionation of the three most active fractions of the first DEAE-cellulose column on a smaller but identical column. The three fractions were combined and returned to KCl-phos by passage over Sephadex G-75 with that buffer. All the protein recovered from the G-75 column was applied with moderate air pressure to an 0.5 X 18 inch DEAE-cellulose column and washed with 50 ml of starting buffer. The same gradient was used to elute but with only 250 ml in each reservoir to start. A single protein peak was eluted which contained all the enzymatic activity, but since all fractions did not have the same specific activity there was no doubt of heterogeneity of the enzyme peak. The two most active fractions, which both had the same specific activity, were approximately two-fold purified over the best fractions of the first DEAE column and contained 22 per cent of the enzyme applied to this last column.

Final ammonium sulfate fractionation.---The above two fractions, which contained slightly over 2 mg protein per ml, were brought to 50 per cent saturation by slow addition of solid ammonium sulfate. The fine precipitate that formed was sedimented at 27,000 X *g* for 30 minutes and dissolved in 2.6 ml of KCl-phos. Sixty per cent of the enzyme was recovered from this step with a 20 per cent increase in specific activity.

A one hundred seventy-fold purification from the starting material had been achieved at this point. Because of the relatively small amount of protein remaining and the apparent detrimental effect of ammonium sulfate further purification steps or crystalization were not attempted.

Enzyme Purity

Ultracentrifugation

Before ultracentrifugation in a Beckman Spinco Model E analytical ultracentrifuge,[1] the one hundred seventy-fold purified preparation was passed through a small Sephadex G-75 column to rid it of residual ammonium sulfate and to change the solvent to 0.1 M potassium chloride, 0.02 M potassium phosphate, pH 7.5. The resulting protein concentration was 5.0 mg per ml. Sedimentation was at 52, 640 rpm and 21°. Photographs were taken at 8-minute intervals after maximum speed was attained and those taken at 26 and 82 minutes are shown in Figure 7. Nearly all the protein sedimented in a single, apparently symmetrical peak, however, upon inspection a slight contaminating peak can be seen running behind the major component. The

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[1] Ultracentrifugation was performed by Mr. Arthur Norris.

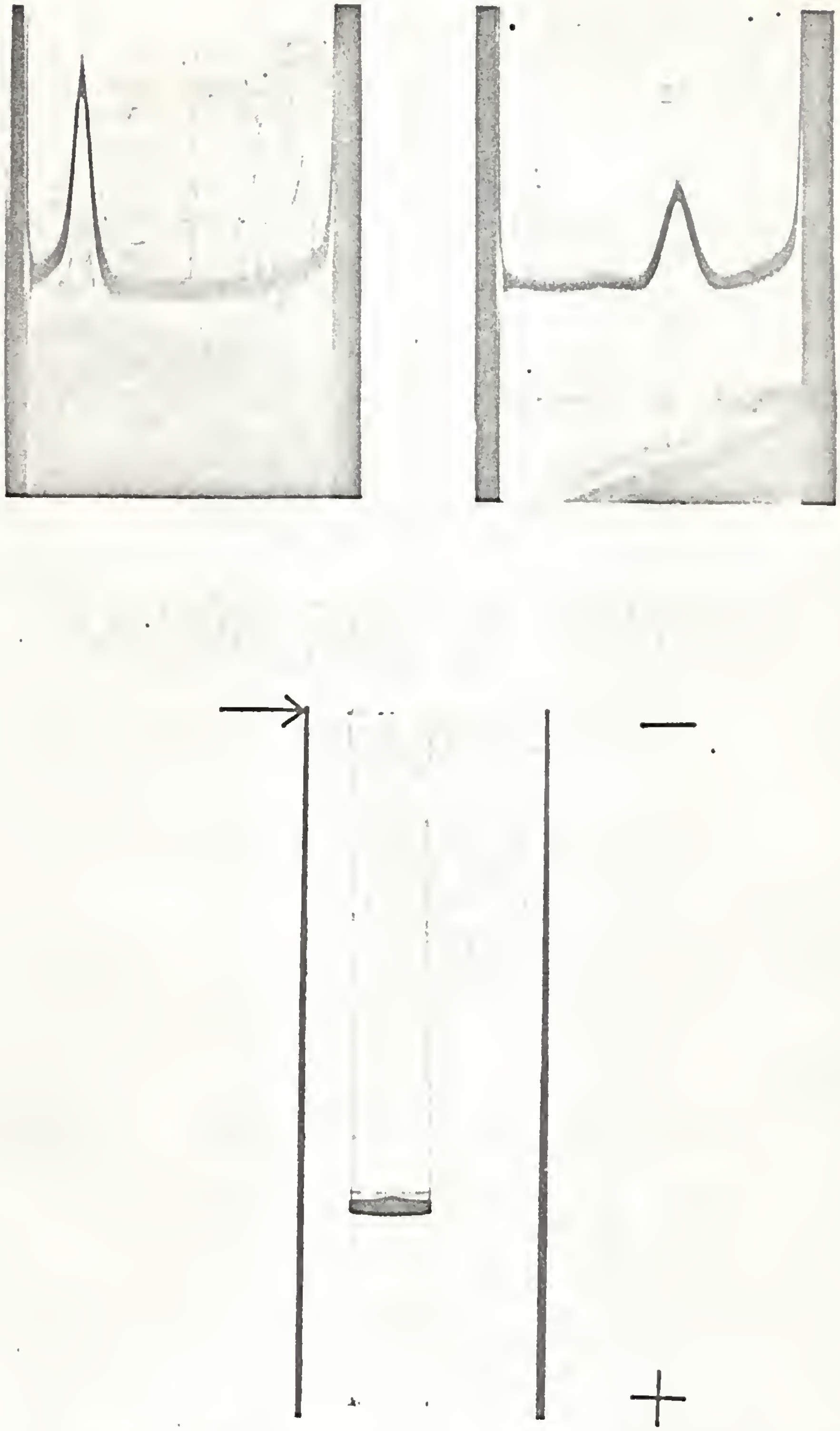

Figure 7. Analytical ultracentrifugation and polyacrylamide gel disc electrophoresis of the final enzyme preparation. Details of the procedures appear in the text.

observed sedimentation coefficient of the major peak was found to be 8.3 S by the graphical method described by Schachman (75). Molecular weight determination by ultracentrifugation has not been attempted.

Recognizing the limitations of analytical ultracentrifugation as a measure of protein purity (especially when gel filtration, a technique which also fractionates proteins on the basis of similar hydrodynamic properties has been used during purification), it was concluded that a large amount of contaminating protein was not revealed in the final preparation by this technique.

Gel electrophoresis

Polyacrylamide gel disc electrophoresis at a running pH of about 9 was accomplished by the method of Clark (76). Fifty µg of the final preparation in 0.2 ml 10 per cent sucrose was applied to the top of replicate gels and subjected to a current of 5 ma per gel. The upper buffer chamber contained 0.005 per cent Brom Phenol Blue which migrated with the buffer and made a "tracking ring" to indicate the progress of the buffer front. The electric field was removed when the buffer front reached the anodic end of the gel Two fast moving bands were evident after staining (Figure 7). The slower moving protein did not appear to represent a large percentage of the total, although quantitative measurements of the relative densities of the two bands were not made.

Attempts at disc electrophoresis at pH 4.3 (77) were unsuccessful. In these experiments the protein migrated through the sample and spacer gels but not beyond the origin (anode) of the 7.5 per cent running gel, even after long times in the electric field. Since the enzyme had precipitated from crude extracts at pH 5.0 it was predicted that it would bear a net positive charge at the lower electrophoretic pH. It was not determined why the protein did not migrate into the running gel in the several experiments conducted.

Starch gel electrophoresis[2] was performed by the method of Smithies (78) at pH 8.5. The same pattern as observed in polyacrylamide gels was obtained by this technique, although the second minor band was more diffuse in the starch gels.

It was assumed that the major ultracentrifugal peak and electrophoretic band were caused by the enzyme under study. By these two analytical criteria the enzyme appeared to be at least 90 per cent pure.

Co-factor Content of the Purified Enzyme

There was little doubt that PLP was a co-factor in the decarboxylation-transamination of AIB. The extent of acetone production from AIB catalyzed by the purified enzyme is shown in Figure 8. As can be seen no acetone was produced when either pyruvate or PLP was excluded from the reaction mixture. The apparent K_{PLP} for this reaction was found to be 5 µM (Table 5). Despite the indication from these data that the co-factor was not tightly bound to the enzyme, B_6 was detected in the final preparation from the spectrum and by microbiological assay. PLP had not been added to the solvent system since gel filtration on G-200. Subsequent to that step the material had been fractionated on DEAE-cellulose twice, precipitated with ammonium sulfate and desalted with Sephadex G-75 twice.

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[2] Starch gel electrophoresis was conducted by Miss Sara Martin.

Enzyme spectrum

The spectrum was taken in a Beckman DU spectrophotometer between 250 and 450 mµ with the purified enzyme at pH 7.5 in 0.1 M potassium chloride, 0.02 M potassium phosphate at room temperature. In addition to the major protein peak at 280 mµ there is a shoulder at 295 mµ and suggestions of broad peaks centered around 330 mµ and 405 mµ. A peak which is not characteristic of free co-factor has been observed between 400 and 430 mµ with nearly all protein -bound PLP systems (28,78) and is presumed to result from a hydrogen bonded aldimine between PLP and the ε-amino of a lysyl residue. The 405 mµ peak observed here suggested this linkage since free co-factor has its highest λ_{max} at 388 mµ. Free PLP shows a weak peak at approximately

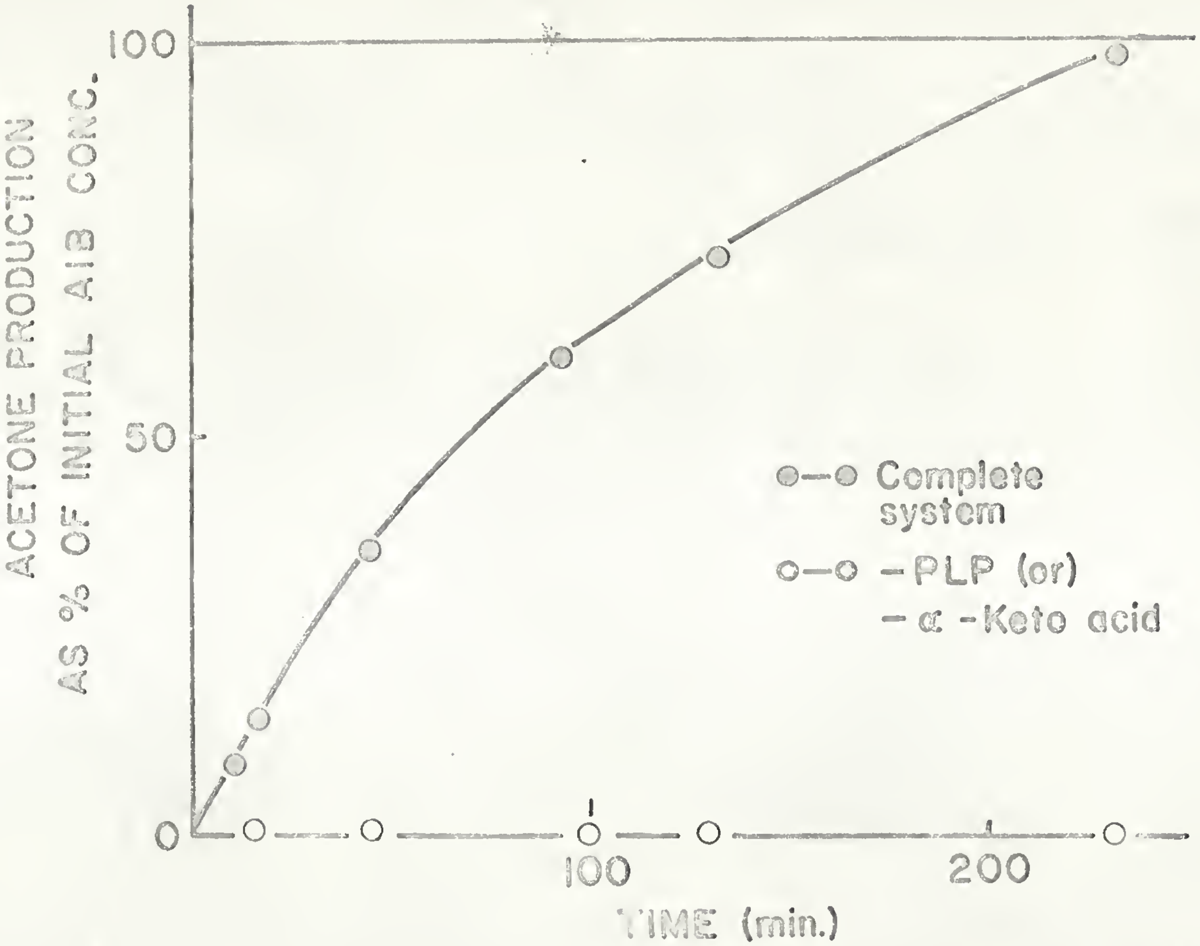

Figure 8. Time course of acetone production in the reaction of AIB with pyruvate catalyzed by the purified enzyme. Complete system contained 20 mM AIB, 20 mM pyruvate, 0.1 mM PLP, 250 mM potassium phosphate pH 7.5. No acetone was detected in reactions from which either PLP or pyruvate had been excluded. Points are averages of duplicate reactions versus non-enzyme controls. The assay for acetone is described in Methods.

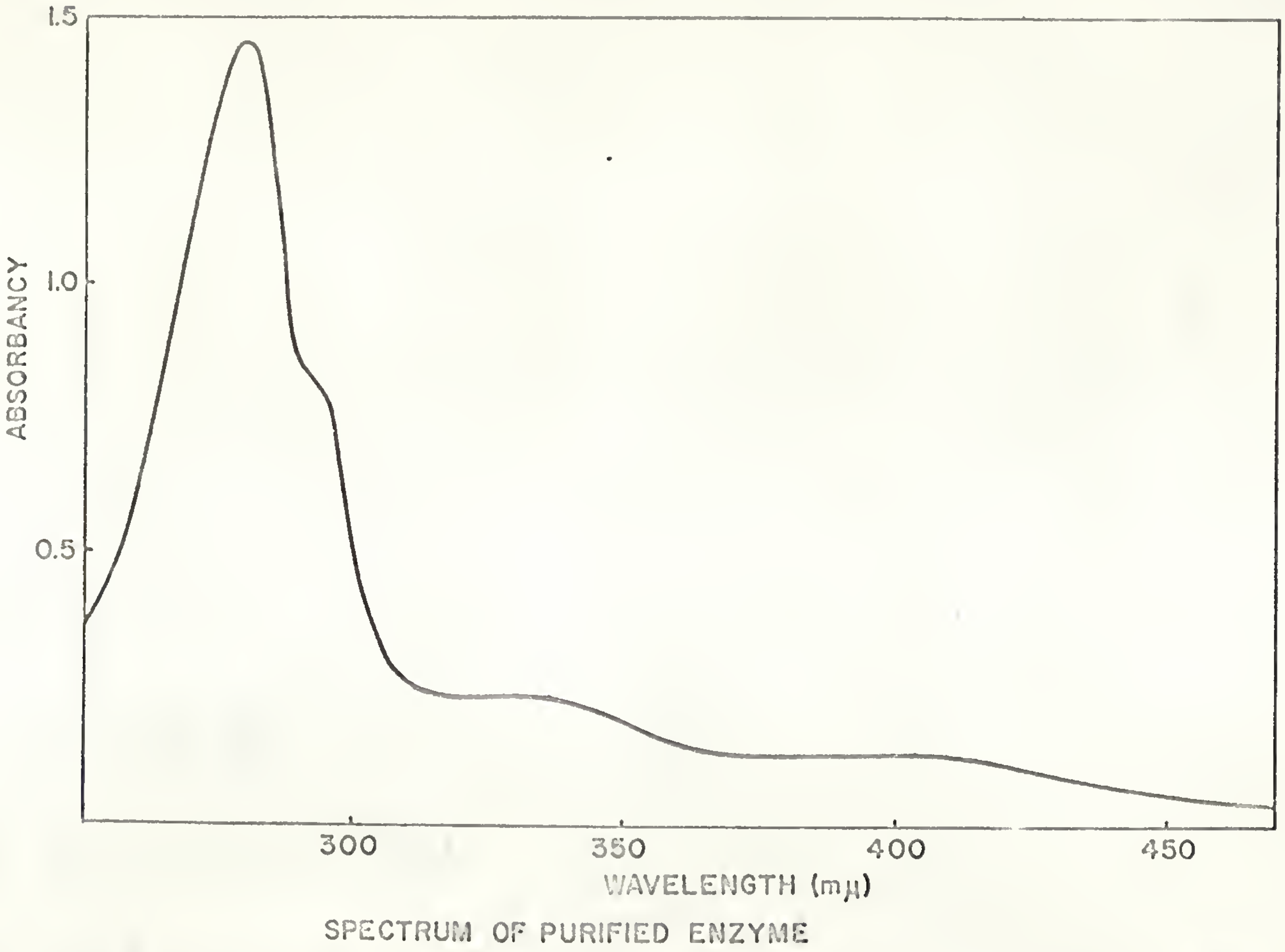

Figure 9. Spectrum of the purified enzyme preparation. Conditions under which the spectrum was taken are described in the text.

337 mμ at neutral pH. A peak close to 330 mμ has been observed in the spectrum of purified glutamic decarboxylase at pH 6.5 (79) but is not generally found in PLP enzymes at neutral pH (28). A peak at 295 mμ is typical of free PLP, but only at pH values below 5.

A detailed spectral study of the enzyme under different conditions of pH and during reaction with different substrates has not been undertaken, but it is warranted since it will surely serve as a useful tool to help reveal the nature of co-factor binding to this enzyme and the steps of the reaction mechanism with different substrates.

Microbiological assay

A sample of the final preparation was appropriately diluted and analyzed for the presence of B_6 by the yeast bioassay of Atkin *et al.* (80).[3] The preparation was found to contain 1 mole of co-factor per 400,000 gm protein. This is presumably PLP although the assay does not distinguish between several of the B_6 vitamers (81).

If one assumes a minimum of 1 mole PLP bound per mole of enzyme and a molecular weight of 150,000 (gel filtration) the yeast assay data imply that the final preparation is a maximum of 40 per cent pure. On the other hand, if co-factor were bound tightly by the enzyme one would expect no requirement for added PLP during assay. In view of the PLP requirement during assay, and the ultracentrifugal and electrophoretic evidence of purity well above 40 per cent, it seems probable that the co-factor became lost from some of the enzyme molecules during purification. Novogrodsky and Meister (82) found that aspartate β-decarboxylase lost up to 80 per cent of its PLP co-factor during purification.

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[3] Dr. W. B. Dempsey performed this assay.

The Reaction Mechanism

The reaction with α-dialkyl amino acids

It appeared obvious that the reaction catalyzed by this enzyme between an α-dialkyl amino acid and pyruvate was a decarboxylation-transamination. However, before the mechanism could be described accurately it was essential that the stoichiometry be determined. One-^{14}C- and 2-^{14}C-DL-isovaline had been synthesized (see Methods) and 1-^{14}C-pyruvate was available, therefore, these substrates were chosen for a study of the details of the reaction. The predicted reaction between isovaline and pyruvate is:

$$\text{DL-Isovaline} + \text{pyruvate} \xrightarrow[\text{Enz}]{\text{PLP}} \text{carbon dioxide} + \text{butanone} + \text{alanine}$$

To identify and quantitate the products three sets of reaction solutions were prepared which were identical in that each contained, in a volume of 0.5 ml, 10 µmole DL-isovaline, 10 µmole sodium pyruvate, 0.1 µmole PLP, and 125 µmole potassium phosphate, pH 7.5, but which differed in that the first set contained 1-^{14}C-DL-isovaline, the second 2-^{14}C-DL-isovaline, and the third 1-^{14}C-pyruvate. All were incubated in appropriate vessels at 30° with or without 21 µg protein added to start the reaction. At the end of 4 hours each set was treated by a procedure which would selectively isolate and quantitate the anticipated radioactive product. The results are shown as Experiment 1, Table 3.

The reaction containing 1-^{14}C-DL-isovaline was assayed for ^{14}C-carbon dioxide. To positively identify the radioactive product from the reaction with 2-^{14}C-DL-isovaline as butanone the entire volume of replicates of this reaction mixture were treated to form butanone 2,4-dinitrophenylhydrazone (see Methods). In this case several additional base washes were used to try to completely remove the 2,4-dinitrophenylhydrazone of pyruvate. The carbon tetrachloride was evaporated and the derivative dissolved in a small volume of ethyl acetate. Aliquots of this sample were chromatographed on silica gel G thin layers in 5 solvent systems with known butanone 2,4-dinitrophenylhydrazone (Figure 10). On 1 chromatogram a second yellow spot appeared. This compound was not checked for radioactivity but may be pyruvate 2,4-dinitrophenylhydrazone. Another aliquot of the ethyl acetate solution was dried onto a planchet, counted in a gas flow counter and found to be highly radioactive. Aliquots from others of this reaction

TABLE 3

Stoichiometry of the Enzyme-Catalyzed Reaction of α-Dialkyl Amino Acids with Pyruvate

Amino Acid Substrate	Exp.	Products as % of Amino Acid Substrate[a]		
		CO_2	Ketone	Alanine
DL-isovaline	1	47	46	42
	2	53	nm[b]	53
	3	nm	80	nm
α-aminoisobutyrate	4	nm	98	nm

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a) Details of assay and methods of identification and quantitation of products are described in the text. Average values of replicate reactions are tabulated.

b) nm = not measured.

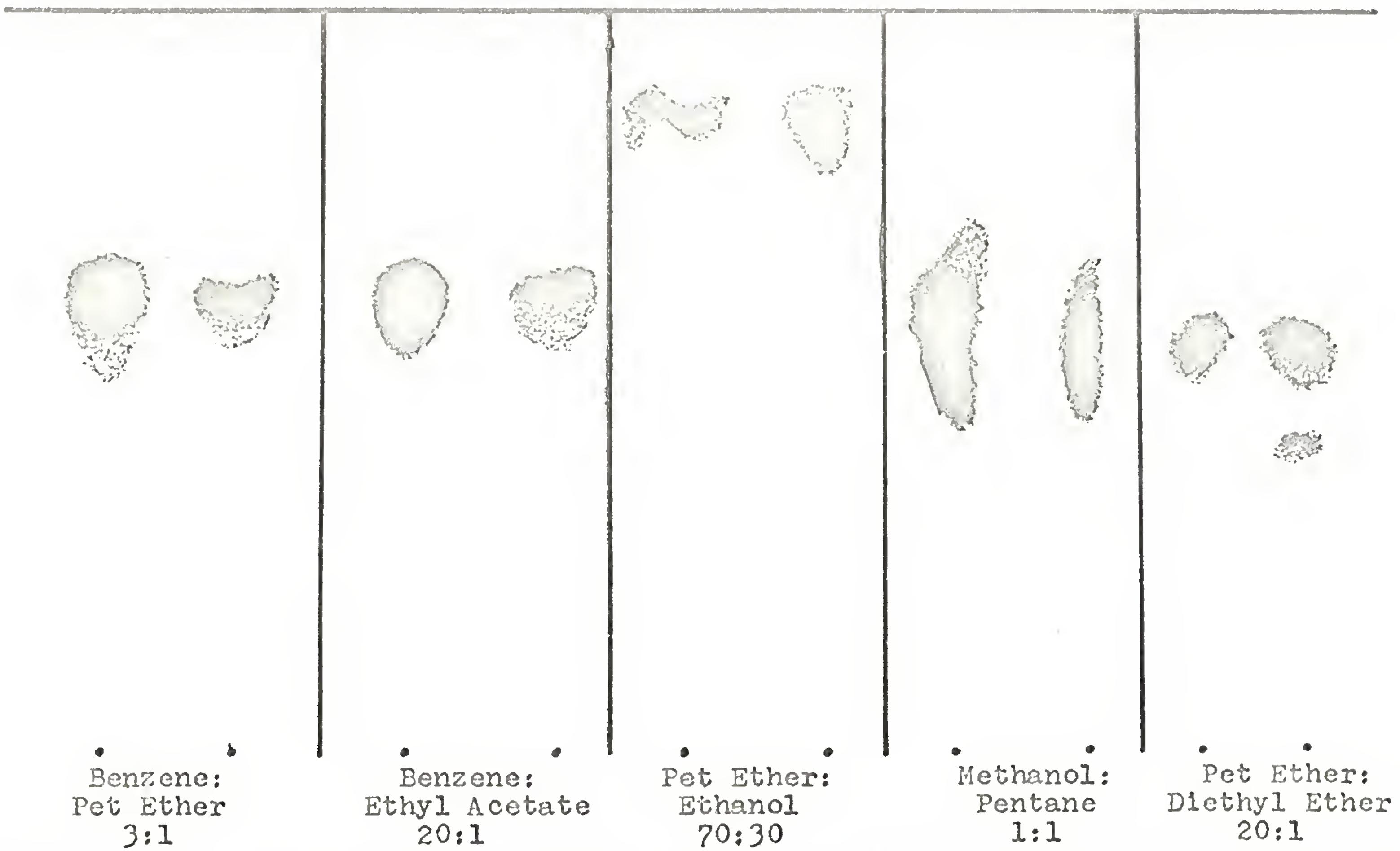

Figure 10. Thin layer chromatograms of 2,4-dinitrophenylhydrazones prepared from butanone and the product of the enzyme-catalyzed reaction between isovaline and pyruvate. Butanone 2,4-dinitrophenylhydrazone was spotted on the left of each plate. The enzyme assay and derivative preparation are described in the text.

set were taken to quantitate the product either by the toluene extraction method or by the absorbancy of its 2,4-dinitrophenylhydrazone. The radioactive product from 2-^{14}C-DL-isovaline displayed a number of properties which reasonably established that it was the predicted ketone.

Replicates from the third set of reaction vessels which initially contained 1-^{14}C-pyruvate were plunged into boiling water to destroy the enzyme and the contents assayed for 1-^{14}C-alanine on Dowex 50 W-X 4 (H^+) columns.

In a separate, but similar, experiment (Experiment 2, Table 3) alanine was positively identified as the amino acid product by quantitative thin layer chromatography (Figure 11). In this experiment the reaction was stopped with 0.1 ml sulfuric acid. Five µl of the reaction mixture was spotted on silica gel G and chromatographed beside alanine and isovaline standards. Alanine and isovaline are readily distinguished on thin layer plates, not only by different R_F values, but by their respective rates of reaction with ninhydrin. Color from alanine appeared on sprayed plates after about 4 minutes' heating at 70°, whereas that from isovaline did not appear until after 7 to 8 minutes at this temperature. Both isovaline and alanine were found in the incubation containing enzyme, while only isovaline was present in the non-enzyme control. The radioactivity which migrated was at the R_F of alanine and was measured. On chromatograms of the non-enzyme control all the radio

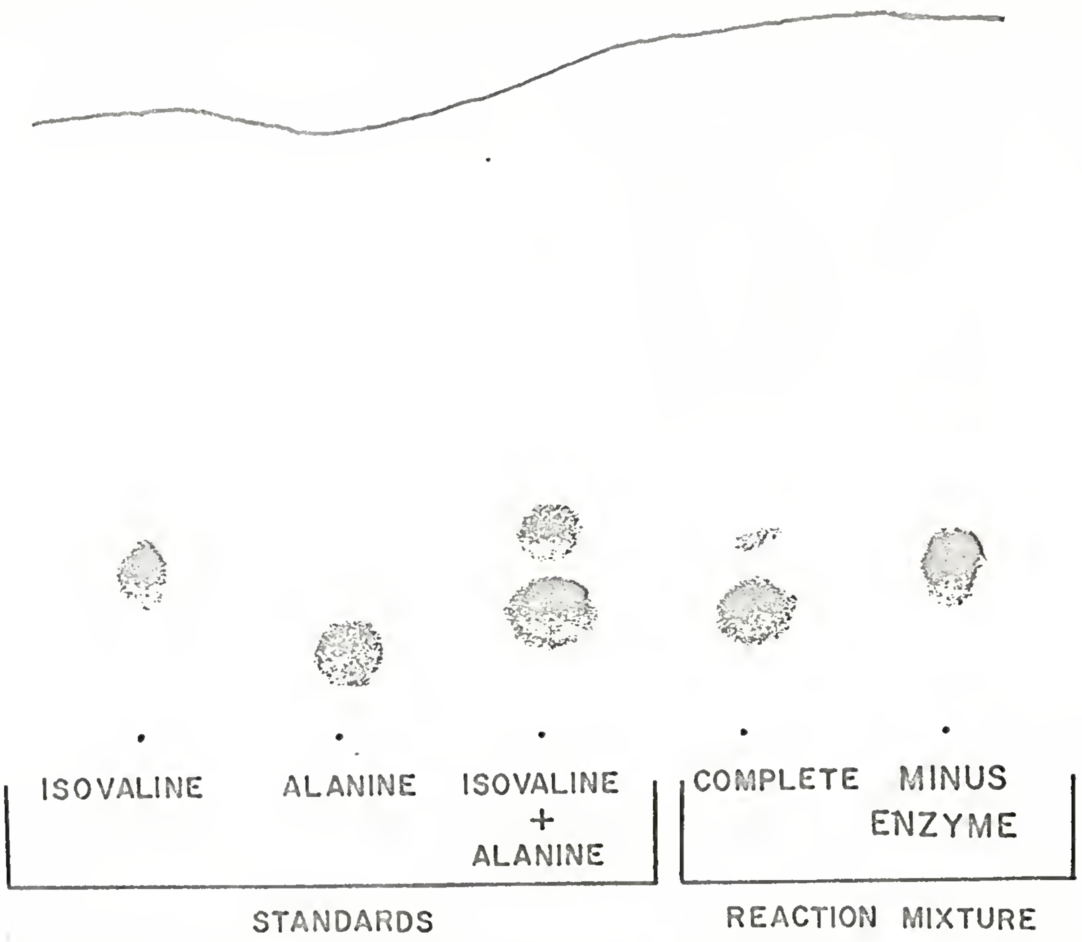

Figure 11. Thin layer chromatograms of isovaline, alanine and reaction mixtures of the enzyme-catalyzed reaction of isovaline with pyruvate. Isovaline and alanine alone and mixed were chromatographed with reaction mixtures and non-enzyme controls. Details of the assay are described in the text.

activity remained at the origin. The quantity of alanine produced as determined by chromatography equalled the amount of carbon dioxide released in identical reactions which contained 1-^{14}C-DL-isovaline to start.

In subsequent experiments only one of the above methods of identification and quantitation was employed to analyze products of α-dialkyl amino acid transamination. In a number of these experiments it was established that the amount of butanone produced was equivalent to the amount of amino group transferred to pyruvate. No detectable carbon dioxide was produced in the absence of the α-keto acid substrate, and in the presence of the α-keto acid substrate α-carboxyl release did not exceed amino group transfer or butanone production. Therefore, decarboxylation without subsequent transamination did not occur to any extent.

Conversion of DL-isovaline in reactions such as those described rarely exceeded 50 per cent, and then only by an amount within the experimental error. Under similar conditions AIB was transaminated up to 98 per cent.(Figure 8 and Experiment 4, Table 3). However, when 10 µmole of 2-^{14}C-DL-isovaline was incubated for 6 hours with a four-fold excess of pyruvate to drive the transamination and a twenty five-fold excess (500 µg) of enzyme to increase the rate of catalysis, over 80 per cent of the radioactivity was extractable into toluene (Experiment 3, Table 3). Thus both enantiomers of isovaline were attacked by the purified preparation. It is likely that one isomer reacted at a slow rate relative to its mirror image which would explain the close to 50 per cent conversion with smaller amounts of enzyme and shorter incubation time.

One-amino-cyclopentane carboxylic acid, the cyclic analog of diethylglycine was also a substrate. This finding was in accord with the observation that both isomers of isovaline were acted upon.

The reaction with α-hydrogen amino acids

As was pointed out in the introduction, the evidence that the mechanism of transamination proceeds sequentially, with conversion of PLP-enzyme to PMP-enzyme by the amino acid substrate followed by regeneration of PLP-enzyme by the α-keto acid substrate, is conclusive. In addition, the two half reactions are independent and fully reversible, such that the amino acid generated from the α-keto acid is also a substrate.

Although the enzyme studied here differs from other amino acid transaminases in that decarboxylation of α-dialkyl amino acids is required to allow formation of the intermediate ketimine, the presumed regeneration step involving pyruvate appears exactly analogous to that of other transaminases. To elucidate the mechanism of this second half reaction the ability of α-hydrogen bearing analogs of the α-dialkyl amino acid to react with pyruvate was measured using the purified preparation. The results of these experiments are shown in Table 4.

After 4 hours' incubation under the same conditions described for Table 3, but with unlabelled pyruvate and 1-^{14}C-L-alanine as substrates, reaction was stopped with acid and carbon dioxide collected (Experiment 1, Table 4). Significantly, only a small amount of radioactivity above the low control values was trapped in hyamine.

When hyamine was suspended over prepared solutions of 1-^{14}C-pyruvate, the same percentage of the radioactivity was trapped in the organic base. Therefore, it appeared that little if any L-alanine was enzymatically decarboxylated and that the radioactivity trapped in hyamine was probably due to non-enzymatic decarboxylation of the 1-^{14}C -pyruvate in acid solution.

However, 26 per cent of the total radioactivity was extracted into ether from the acidified reaction mixture. Essentially no pyruvate could be extracted from neutral or basic reaction mixtures. One-^{14}C-alanine was not extractable at any pH. The fact that the extracted radioactive compound was acidic and contained the α-carboxyl of alanine led to the necessary conclusion that it was pyruvate.

When radioactive pyruvate was a substrate, 1-^{14}C -alanine was produced as determined by thin layer chromatography and ion exchange columns. Thus within the limits of detection the only reaction catalyzed in these

TABLE 4

Stoichiometry of the Enzyme-Catalyzed Reaction of α-Hydrogen Bearing Amino Acids with Pyruvate

Amino Acid Substrate	Exp.	Products as % of Amino Acid Substrate[a]		
		CO_2	α-Keto Acid	Alanine
L-alanine	1	<0.5	26	nm[b]
	2	nm	nm	39
D-alanine	3	<0.3	<0.5	nm
	4	nm	nm	<2
DL-α-amino-*n*-butyrate	5	<0.4	29	35
L-α-amino-*n*-butyrate	6	nm	nm	11
D-α-amino-*n*-butyrate	7	nm	nm	<0.3

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a) Details of assay and methods of identification and quantitation of products are described in the text.

b) nm = not measured.

experiments was transamination between L-alanine and pyruvate potentiated by loss of the α-hydrogen of L-alanine.

Minute amounts above the non-enzyme control values of acid volatile and acid extractable radioactivity were generated in the reactions with 1-^{14}C-D-alanine as amino acid substrate (Experiment 3, Table 4), and minor amounts of ^{14}C-alanine could be detected by the ion exchange technique when unlabelled D-alanine and 1-^{14}C-pyruvate were substrates (Experimental 4, Table 4). The possibility that D-alanine may be decarboxylated at a very slow rate requires further testing, but on the basis of the results of these experiments it can be concluded that D-alanine was neither decarboxylated, nor transaminated to pyruvate, at a rate comparable to that of other substrates.

L- but not D-α-amino-*n*-butyrate, an α-hydrogen bearing analog of isovaline, was transaminated to α-keto butyrate (Experiments 5-7, Table 4). The small amount of radioactivity appearing as carbon dioxide when 1-^{14}C-DL-α-amino-*n*-butyrate was used could be explained as when 1-^{14}C-L-alanine was the substrate.

The results of the experiments outlined in Tables 3 and 4 indicated that the same enzyme accomplished transamination of α-dialkyl amino acids by initial cleavage of the α-carboxyl group necessarily, and transamination of the L-isomers of α-hydrogen bearing analogs by cleavage of the α-hydrogen preferentially. Although the final preparation with which these experiments were performed appeared reasonably pure, it was essential to determine by other means whether this indication was true.

Evidence for a Single Transaminase

Relative rates of α-carboxyl and α-hydrogen labilizing transamination

Certain of the apparent kinetic properties have been determined with the enzyme for several of the substrates (Table 5). Apparent K_m values and V_{max} values were obtained from double reciprocal plots (83) with essentially saturating concentrations of the second substrate and the co-factor. Turnover numbers were calculated from V_{max} values assuming the final preparation to be a pure enzyme of 150,000 molecular weight. Under identical conditions the V_{max} for L-alanine transamination with pyruvate was 2.3 times that for decarboxylation-transamination of AIB with pyruvate. Therefore, if the α-hydrogen labilizing enzyme was a trace contaminant of the α-dialkyl transaminase, this enzyme must have a much greater turnover number than the latter.

Several more direct approaches were taken to test the identity question.

Relative reactivity of α-keto acid substrate

First, since the amino acid substrate presumably is not involved mechanistically in the half reaction of

TABLE 5

Apparent Kinetic Constants for Substrates of the Purified Enzyme Preparation

Substrate	Apparent K_m (m*M*)	Turnover Number
Pyridoxal 5'-phosphate	0.005[a]	
Pyruvate	2[b]	
α-aminoisobutyrate	8[c]	1550[c]
L-alanine	33[c]	3600[c]

pH Optimum for AIB, Pyruvate = 8.0 - 8.5[d]

[a] 20 m*M* pyruvate, 20 m*M* AIB, or L-ala, 50 m*M* potassium phosphate, pH 7.5, using purified preparation.

[b] 20 m*M* AIB, 0.1 m*M* PLP, 50 m*M* potassium phosphate, pH 7.5, using 100,000 X *g* extract.

[c] 20 m*M* pyruvate, 0.1 m*M* PLP, 50 m*M* potassium phosphate, pH 7.5, using product from ion exchange (Figure 14) with specific activity of 5.6. Turnover calculations assume pure enzyme has specific activity of 10.0 and molec. wt. = 150,000.

[d] potassium phosphate, pH 5.7 - 8.4; sodium pyrophosphate, pH 7.9 - 9.5, using purified preparation.

PMP-enzyme with the α-keto acid, one would expect, if a single enzyme were responsible, the same relative efficiency of different α-keto acid substrates for both transaminations. Using the purified preparation and identical reaction conditions, but with isovaline the substrate in one set and L-alanine the substrate in the second, this correlation was observed with all α-keto acids tested (Table 6). However, it was noted that, compared with pyruvate, the ability of all other α-keto acids to transaminate L -alanine appeared considerably poorer than their ability to transaminate isovaline. This is understandable in view of the apparent irreversibility of the α-dialkyl transamination but ready reversibility of L-alanine pyruvate exchange (below). Thus, even though reactions were allowed to proceed less than a few per cent, the fact that pyruvate, which was generated from L-alanine, was a much more active α-keto acid substrate than the one being tested would reduce the measured extent of 1-^{14}C-L-alanine transamination in all assays but the one in which pyruvate itself was being tested.

Variable activity was observed with oxaloacetate even when freshly made solutions were used. However, since no measurable transamination of L-aspartate with 1-^{14}C-pyruvate could be detected in another experiment, it was suspected that the activity measured with oxaloacetate was

TABLE 6

Relative Rates of Transamination of Isovaline and L-Alanine with Several Different α-Keto Acid Substrates[a]

α-Keto Substrate	Relative Rate	
	DL-Isovaline[b]	L-Alanine[c]
Pyruvate	1.00	1.00
α-keto butyrate	0.95	0.65
α-keto valerate	0.49	0.14
Glyoxalate	0.12	0.09
α-keto isocaproate	0.06	0.03
α-keto phenylpyruvate	0.00	0.00
α-keto glutarate	0.00	0.00

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a) Each reaction contained 20 m*M* α-keto acid, 45 m*M* amino acid, 0.1 m*M* PLP, 50 m*M* potassium phosphate, pH 7.5. The product from ion exchange(Figure 14), was the enzyme source. Relative rates were calculated from the mean of duplicate reactions.

b) Assayed by toluene extraction of 2-^{14}C-butanone produced from 2-^{14}C-DL-isovaline.

c) Assayed by ether extraction of 1-^{14}C-pyruvate formed from 1-^{14}C-L-alanine.

due to pyruvate which arose from decarboxylation of the β-keto acid, and that oxaloacetate itself was not an amino acceptor. It was also notable that the other most common amino acceptor, α-keto glutarate, was inactive with both amino acid substrates.

Heat stability

As a test of similarity of protein structure the heat stability of the α-hydrogen and α-carboxyl labilizing activities was determined with the purified preparation. An aliquot of the concentrated protein solution was diluted with KCl-phos and divided into 1 ml samples. These were heated separately in water baths at different temperatures; 35 minutes at 53°, and 5 minutes at 60, 65, and 70°. After heating, each solution was rapidly cooled to below 5° and the activity with AIB and L-alanine measured. The denaturation profiles for both substrates are shown in Figure 12, and coincide.

Gel filtration and ion exchange

The elution patterns of protein and of α-hydrogen and α-carboxyl labilizing activities from Sephadex G-200 and DEAE-cellulose columns are shown in Figures 13 and 14. The preparation used for the gel filtration experiment consisted of pooled, low activity, fractions from a previous Sephadex G-200 column. The active fractions from the G-200 column represented in Figure 13 served as the sample for the ion exchange column of Figure 14.

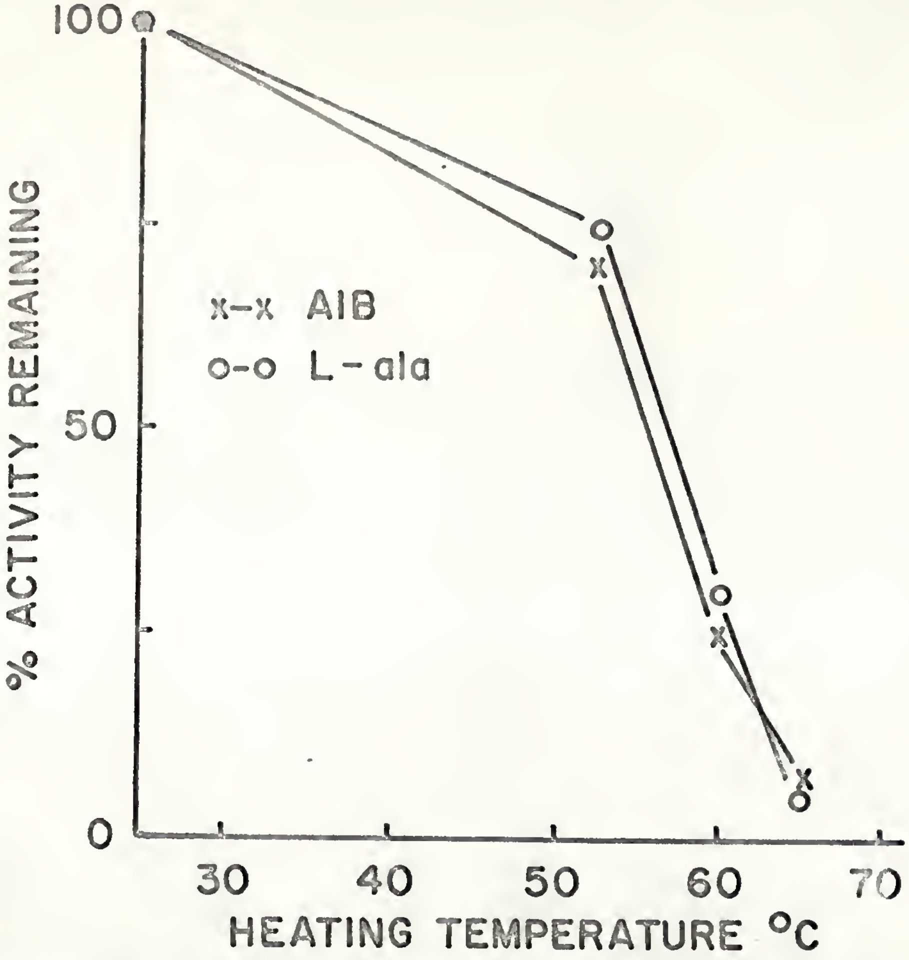


Figure 12. Heat stability of the α-carboxyl and α-hydrogen labilizing activities in the purified preparation, AIB transamination with pyruvate was measured by acetone production. One-^{14}C-L-alanine transamination with pyruvate was measured by ether extraction of 1-^{14}C-pyruvate formed. Points are averages of duplicate assays.

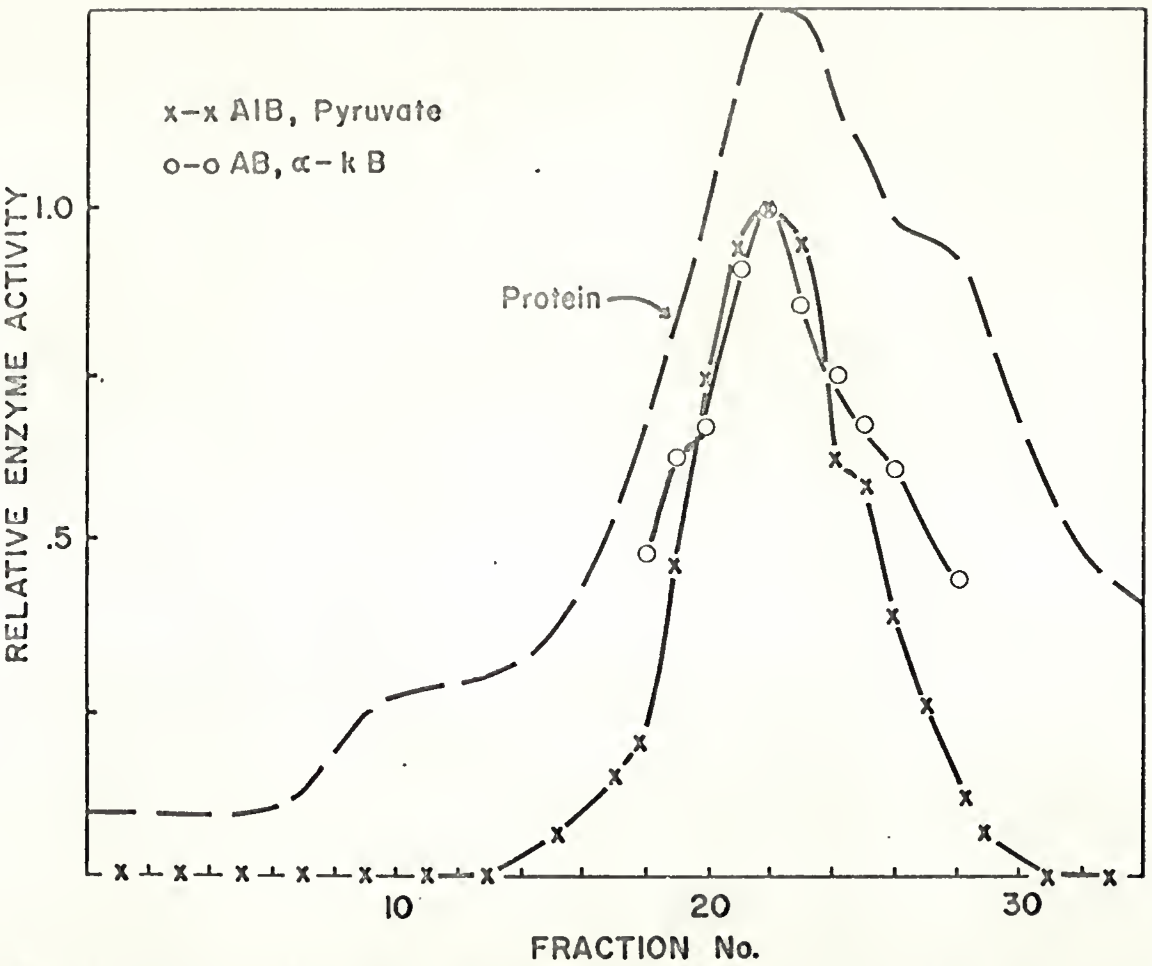


Figure 13. Gel filtration through Sephadex G-200 of α-carboxyl and α-hydrogen labilizing activities. The activity found in each 4.0 ml fraction is plotted relative to that of the most active fraction which is assigned unit activity. The enzyme sample and assays are discussed in the text.

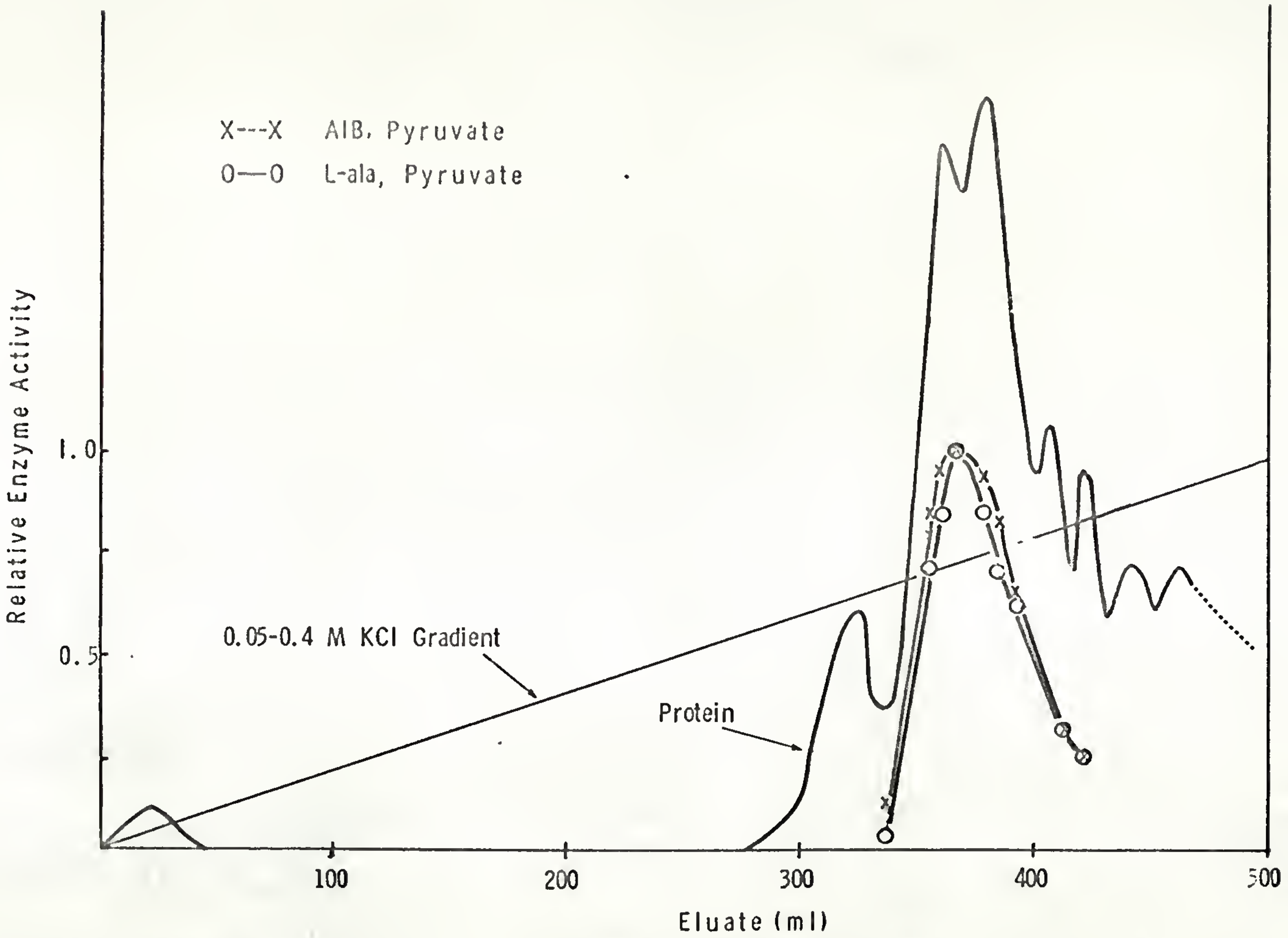


Figure 14. Gradient elution of α-carboxyl and α-hydrogen labilizing activities from DEAE-cellulose. Activity in each 6.0 ml fraction is plotted as in Figure 13. Details of chromatography and enzyme assay appear in the text.

The relative activity of the two transaminations was the same in all fractions within the error limits of the assay used. It had been considered likely that Ps-1 would contain more than one enzyme capable of alanine pyruvate transamination, even if the α-dialkyl transamination acted also on L-alanine. The presence of any of this other enzyme in AIB transaminating fractions would have led to ambiguous results. One-^{14}C-DL-α-amino-n-butyrate and α-keto butyrate were used as the α-hydrogen amino acid and α-keto acid substrates, hoping that L-α-amino-n-butyrate would not be a good substrate for such a contaminating transaminase. However, the assay for α-amino-n-butyrate transamination involved elution and quantitation of ^{14}C-α-keto butyrate from a Dowed 50 (H^+) column, a procedure which, because of dilution, was inaccurate for rate studies when low activities were present. The more accurately assayed alanine pyruvate transamination was employed to measure α-hydrogen labilizing activity eluted from the DEAE-cellulose column, and closer correlation of the two activities was observed. More than one α-hydrogen labilizing transaminase did not appear to be present in the starting sample. The conclusion from these data was that the enzyme responsible for both activities had similar size and charge characteristics.

Co-induction of α-hydrogen and α-carboxyl labilizing activities

Both α-hydrogen and α-carboxyl labilizing activities were present in extracts of cells induced with either DL-isovaline or DL-α-amino-*n*-butyrate in the absence of other nitrogen sources, while both activities were greatly reduced in cells grown with ammonia as nitrogen source. However, the ratio of α-hydrogen to α-carboxyl labilizing activity was greater in extracts of the Dl-α-amino-*n*-butyrate grown cells. The possibility of such a finding was anticipated since a second α-hydrogen labilizing transaminase with somewhat different specificity might be induced by the α-hydrogen bearing amino acid. It was for this reason that the better substrate, but more common amino acid, L-alanine; had not been used as the α-hydrogen bearing inducer.

The results of all the experiments described in this section imply that one enzyme was responsible for both types of transamination. There was no evidence contrary to this interpretation and it was therefore concluded that the purified preparation contained a PLP-dependent transaminase which proceeded by different mechanistic steps with α-dialkyl amino acids than with α-hydrogen bearing amino acids.

Other Details of the Reaction Mechanism

Stereochemistry of the amino acid product

Before the reaction mechanism of this transaminase could be accurately described it was essential to establish the stereochemistry of the amino acid product, although the L isomer was expected. This question was answered by a direct and an indirect test.

First, the labelled alanine produced upon incubation of AIB and 1-^{14}C-pyruvate with enzyme was isolated from a Dowex 50 (H^+) column, concentrated on a steam bath and used as a substrate for the enzyme, which had been shown to be specific for the L isomer. However, less than the calculated amount of labelled alanine exchanged with pyruvate in this second incubation. The result allowed the conclusion that L-alanine was a product, but did not allow the exclusion of D-alanine as a product, also. Rather than pursue this type of proof, a second means was employed to test whether only the L isomer was a product.

If both the substrate and product amino acids had the same configuration, the rate of an exchange reaction between alanine and pyruvate would remain constant throughout a given incubation; for, as each alanine molecule was converted to pyruvate, a pyruvate would be converted to alanine of the substrate form, so that the concentrations of all substrates would remain constant. In addition, as was pointed out by Jenkins and Sizer (48), if one of these species was labelled at the outset of exchange, a first order reversible equilibration of isotope would occur, such that at equilibrium the specific activity of both substrates would be the same, provided they were both present in the same initial concentration. Jenkins and Sizer showed further that a semi-log plot versus time of the decrease of radioactivity to the equilibrium value had a slope which was proportional to the true rate of the exchange reaction. However, the slope of this plot would be constant only if the product amino acid was an equally active substrate.

The exchange reaction between equal amounts of ^{14}C-L-alanine and pyruvate catalyzed by α-dialkyl amino acid transaminase is shown in Figure 15. Figure 15 A shows that at equilibrium 50 per cent of the radioactivity exists as pyruvate. A semilog plot of the loss of radioactivity from alanine as equilibrium was approached versus time (Figure 15 B) was linear and therefore established that the product amino acid was the predicted L-alanine.

The irreversibility of the α-carboxyl labilizing half reaction

Reversibility of the half reaction involving cleavage of the α-carboxyl of the α-dialkyl amino acids was tested by attempting to measure exchange between isovaline, ^{14}C-carbon dioxide and butanone. Replicate reaction mixtures

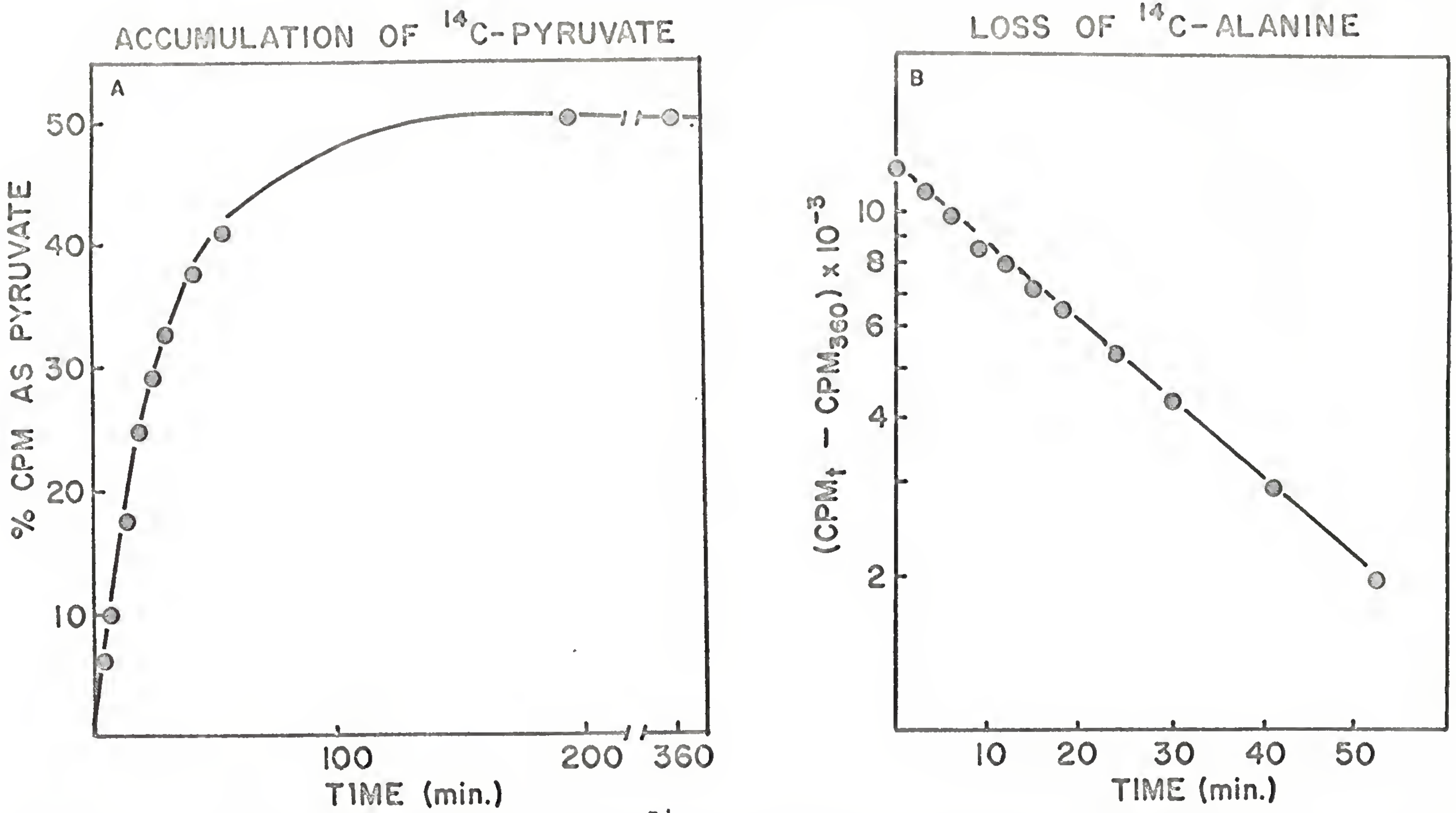


Figure 15. Exchange between 60 mM 1-^{14}C-L-alanine and 60 mM pyruvate, pH 7.5. Assay was by ether extraction of 1-^{14}C-pyruvate. Points are averages of duplicate reactions.

were prepared which contained in 1.0 ml, 20 µmole each of DL-isovaline, sodium ^{14}C-bicarbonate (1.5×10^6 cpm), and butanone; 0.5 µmole PLP, and 50 µmole potassium phosphate, pH 7.5. At the end of 6 hours' incubation with 270 µg enzyme, carbon dioxide was liberated with sulfuric acid and trapped in hyamine. An aliquot of the carbon dioxide free reaction mixture was counted to detect any ^{14}C which may have become incorporated into isovaline during the incubation.

In a second experiment carbon dioxide exchange with the co-factor bound intermediate (i.e. reversibility of the presumed aldimine to ketimine, α-carboxyl cleavage step) was tested analogously except that butanone was excluded from these reaction mixtures which however, contained 0.2 µmole pyruvate per ml. Pyruvate was included to reconvert to PLP-enzyme, the PMP-enzyme which would be formed and be incapable of the exchange reaction in question.

In both experiments the radioactivity remaining in the reaction solutions after liberation of carbon dioxide was equal to background indicating no detectable fixation of ^{14}C.

DISCUSSION

The Reaction Mechanism

The overall reaction mechanism for the α-dialkyl amino acid transaminase, constructed from the evidence presented in the preceding section is summarized in Figure 16. Accepted structural notations for major intermediates presumed to exist in PLP-catalyzed decarboxylation and transamination reactions have been used to illustrate the mechanism. It is written as the reaction of an α-dialkyl amino acid with an α-keto acid.

Cleavage of the α-carboxyl group of the aldimine (I) leads to formation of the ketimine (II) which becomes protonated at the formyl carbon to produce the less conjugated ketimine (III). This intermediate is hydrolyzed to produce a ketone and PMP-enzyme. The mechanism of the first half reaction appears to be the enzymatic analog of the non-enzymatic decarboxylation-transamination shown in Figure 2.

The second half reaction involving PMP-enzyme and the α-keto acid is identical with other amino acid transamination mechanisms (Figure 3), in that it is fully reversible. As such it presumably includes reversible tautomerization of the ketimine (IV) to the aldimine (VI) via the fully conjugated intermediate (V).

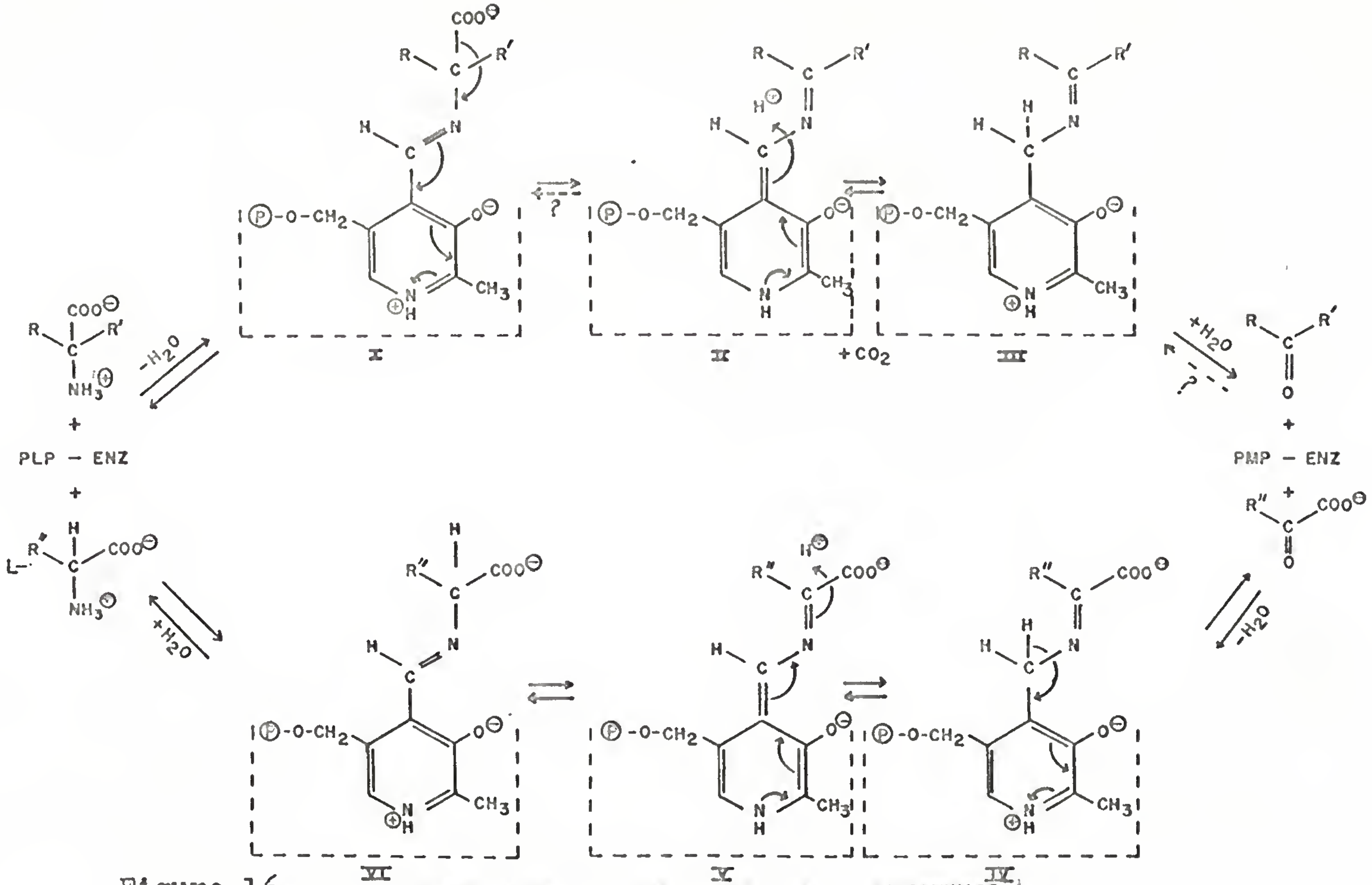


Figure 16. PROPOSED MECHANISM OF α- DIALKYL AMINO ACID TRANSAMINASE.

The enzyme did not catalyze detectable exchange of the α-carboxyl of isovaline with ^{14}C-bicarbonate or exchange between isovaline, butanone and ^{14}C-bicarbonate. The reversibility of the hydrolysis of intermediate III was not determined. Although the equilibrium constant for the overall reaction of an α-dialkyl amino acid transamination with pyruvate was not measured, it is evident from the extent of reaction with AIB (Figure 8) the equilibrium strongly favors decarboxylation.

The unique feature, which distinguishes this enzyme from other PLP-dependent enzymes that have been described is its ability to transaminate amino acids with a tertiary α-carbon by cleavage of the α-carboxyl group. However, the most significant feature of the mechanism in terms of its relationship to our understanding of PLP-dependent enzyme mechanisms and reaction specificity would seem to be the ability to potentiate transamination of α-hydrogen bearing analogs of these amino acids by cleavage of the α-hydrogen in preference to the α-carboxyl. Because such a property could be significant to the construction of concepts about the factors controlling reaction specificity of this, and possibly other B_6 enzymes, it is important to consider the justification of this conclusion from the experiments conducted to test the question.

The experiments that were performed were designed to reveal physical or chemical differences between the proteins or catalytic sites involved in the α-hydrogen and α-carboxyl labilizing transaminations. The results of these experiments indicated that the enzyme which transaminated α-dialkyl amino acids had the following properties in common with that responsible for alanine-pyruvate exchange:

1) simultaneous induction by the same compounds, with either substrate configuration about the α-carbon;
2) the same size, charge, and denaturation properties as measured by gel filtration, ion exchange, and heat stability;
3) the same degree of co-factor requirement during assay; and
4) the same relative activity with a variety of α-keto acid substrates and no activity with α-keto glutarate and probably oxaloacetate, the two most common amino acceptors.

If two enzymes were involved one would be required to explain why a transaminase which was able to tautomerize the pyruvate-PLP ketimine intermediate to the aldimine (as in the formation of alanine during regeneration of the PLP form of the α-dialkyl transaminase) was unable to catalyze the reverse tautomerization to form the same ketimine. The evidence seemed overwhelming in favor of the conclusion that a single enzyme was responsible for both reactions.

The Dependence upon Added PLP for Transaminase Activity

The apparent K_{PLP} for α-dialkyl amino acid transaminase was approximately 5 µM, yet the final preparation contained B_6 even after the enzyme had undergone several procedures during purification which should have removed free PLP or a weakly bound co-enzyme. It is well understood that K_m can not be interrupted as an indication of binding, but if there is independent evidence, as in this case, that the co-factor remained bound to its active site, one can expect a low K_m or even no requirement for added co-factor during assay.

There are at least two possible explanations for the findings with this enzyme. First, since 50 µM PLP was added during the early purification steps, the co-factor presumably bound to protein in the final preparation may have become attached, during purification, to free amino groups on the protein (84) and have, in fact, no connection with the co-factor requirement for catalysis. Thus the co-enzyme binding site could be essentially empty in the final preparation. The weak peak in the spectrum of the purified enzyme (Figure 9) about 405 mµ is suggestive of PLP present as an aldimine.

On the other hand, the requirement for added PLP during catalysis could reflect the difference in binding properties of PLP and PMP since the latter is formed during the transamination reaction. In several experiments with the α-dialkyl transaminase in which enzyme was used in great excess (270 µg) of the normal concentration, it was noted, incidentally, that after long incubation times the bright yellow color of the added PLP (500 µM) became bleached implying possible conversion of most of the PLP originally present to PMP, free in solution. Such a loss of PMP from the active site after transamination is an extreme example of the type of inactivation observed of aspartate β-decarboxylase (82) when PLP or an α-keto acid were not present during assay.

It is predicted then, that PMP will fulfill the co-factor requirement for the α-dialkyl amino acid transaminase but will have a much higher K_m than PLP. One-half the maximum activity possible with 50 µM PLP was observed in an assay in which 200 µM PMP was used. However, it was discovered by other workers in this laboratory that the PMP preparation used contained 4 per cent PLP as a contaminant; this made the solution 8 µM in PLP, a concentration sufficient to account for the observed activity. Further experiments with pure PMP will be required to test its ability to serve as co-factor. PAL at 200 µM was inactive.

The Natural Substrate

The findings in this study imply that, in nature, this transaminase could have as its usual substrates α-dialkyl amino acids and pyruvate, and functions to assimilate the nitrogen of these or similar "unnatural" amino acids into the mainstream of intermediatry metabolism. It has been reported that AIB is found in the protein of horse muscle (85), and as a component of an antibiotic (86). Confirmation of either of these reports could justify the existence of a bacterial enzyme designed specifically to degrade these compounds.

Further, 1-aminocyclopentane carboxylic acid which, in space, has a structure closely resembling both D and L-isovaline, is a substrate for the enzyme. Reasoning from this, it should be the case that 1-aminocyclopropane carboxylic acid, the cyclic analog of AIB, and a natural component of some fruits (87,88), should also be a substrate.

Inducibility by α-hydrogen amino acids implies a use in the cell of the enzyme for their transamination. Yet L-alanine exchange with pyruvate was the only reasonably rapid reaction observed with the α-hydrogen substrates and the apparent K_m for alanine was much greater than for AIB (Table 5).

Conversely the apparent K_m for pyruvate was lowest of all substrates tested. The specificity indicated by the several amino and α-keto acids tested, appears to be limited to compounds with short non-polar α-R groups. The rate of transamination decreased considerably when the side chain length of the α-keto acid substrate was increased from two to three carbons as in the shift from α-keto butyrate to α-keto valerate (Table 6). The rate decreased more sharply when the alkyl group was removed altogether, as in the shift from pyruvate to glyoxalate. The aromatic side chain of phenyl pyruvate and the terminal carboxyl of α-keto glutarate prevented activity with these compounds. Likewise, L-aspartate was not an amino acid substrate. It should be a safe assumption, in view of the evidence for reversibility of this and all other amino acid transaminases which have α-hydrogen amino acid substrates, that the amino acid corresponding to a particular α-keto acid will show reactivity proportional to that shown by the α-keto acid and vice versa. The only class of common amino or α-keto acids not tested were those with positively charged side chains. Unless, one of these is a good substrate, the utility of this enzyme as a strictly α-hydrogen amino acid transaminase is questionable.

Control of Synthesis of the α-Dialkyl Amino Acid Degrading System in Ps-1

α-Dialkyl amino acid transaminase was induced in Ps-1 by DL-isovaline, AIB and 1-aminocyclopentane carboxylic acid, as well as the α-hydrogen bearing analog of isovaline, DL-α-amino-n-butyrate. Induction by alanine was not measured. There was no evidence of coordinate or sequential induction of a pathway for the complete degradation of α-dialkyl amino acids since the organism was unable to utilize the ketone products for carbon. However, the enzyme exhibited some physical properties (e.g., sedimentation coefficient, heat stability) in common with the enzyme studied by Aaslestad and Larson (11), which came from a bacterium that used AIB for carbon. Both organisms were tentatively assigned to the genus Pseudonomas. It could be that the bacterium used in this laboratory was closely related to their organism but had lost, through mutation, the ability to degrade the ketones. Or possibly the natural "unnatural" amino acid substrate has not been discovered but is one which could be degraded completely by this organism.

The control of this enzyme may be of interest because unlike most inducible, degradative enzymes, it has two substrates, the amino and the α-keto acid. It is assumed that induction requires only the amino acid substrate; however, a simultaneous need for an α-keto acid for induction can not be excluded since glucose, which is supplied to this organism for growth, is readily broken down to pyruvate. On the other hand, if one defines the primary function of the enzyme to be the transfer of nitrogen from uncommon α-dialkyl amino acids to pyruvate, then one may anticipate that an amino acid substrate alone would be both necessary and sufficient for induction.

On the Nature of the Reaction Specificity

PLP-aldimine complexes oriented for cleavage of the α-hydrogen (I), the α-R group (II) and the α-carboxyl (III) according to the model proposed by Dunathan (57) are shown in Figure 17. Certain predictions were made by him from this model.

Dunathan proposed that if the binding of the carboxylate anion established the orientation of the bond to be cleaved perpendicular to the PLP ring, one might expect to find cases of a dual reactivity with certain enzymes if an amino acid of opposite configuration were allowed to bind in the active site with a different bond oriented for cleavage. The observation (89) of a slow transamination of D-alanine by L-serine transhydroxymethylase was sited as an example. The orientation of L-serine and D-alanine at the active site of this enzyme would be as shown in Figure 18. As can be seen, the binding of the carboxyl in the PLP-aldimine complex places the α-methylol

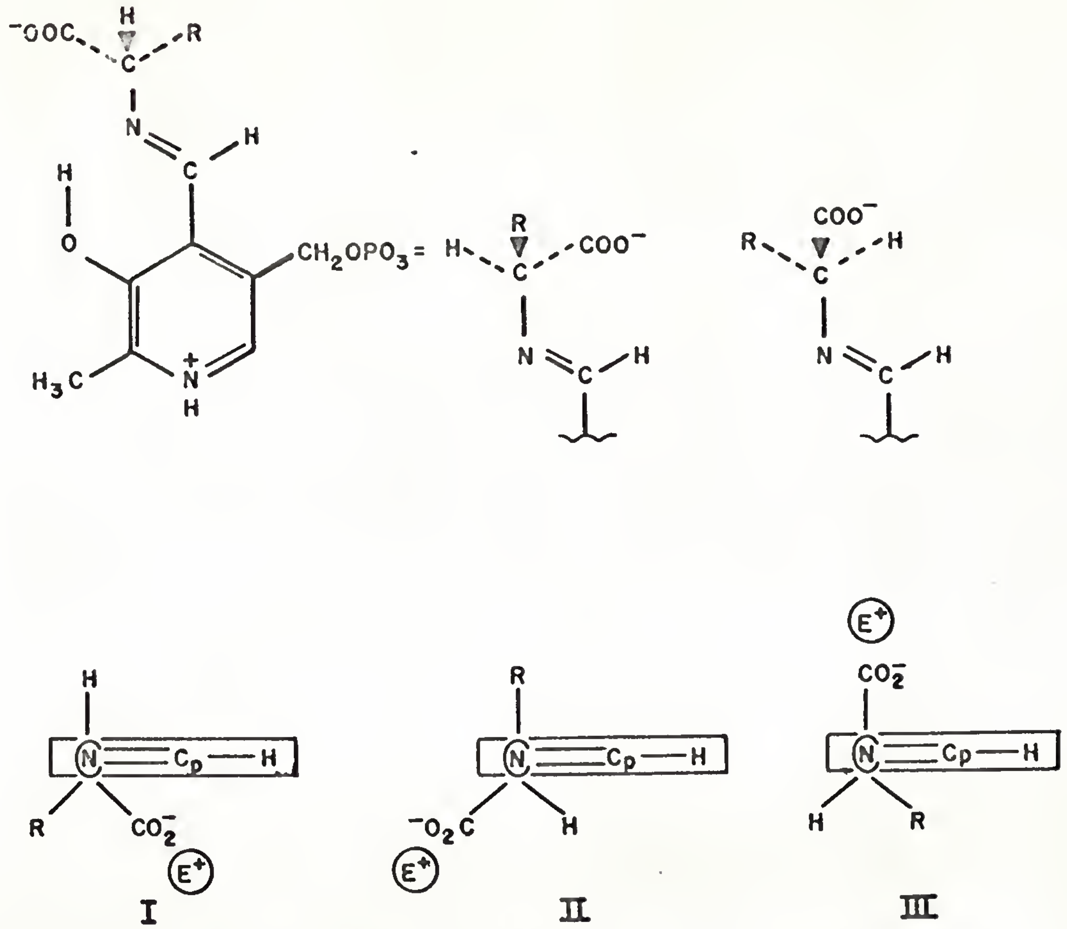
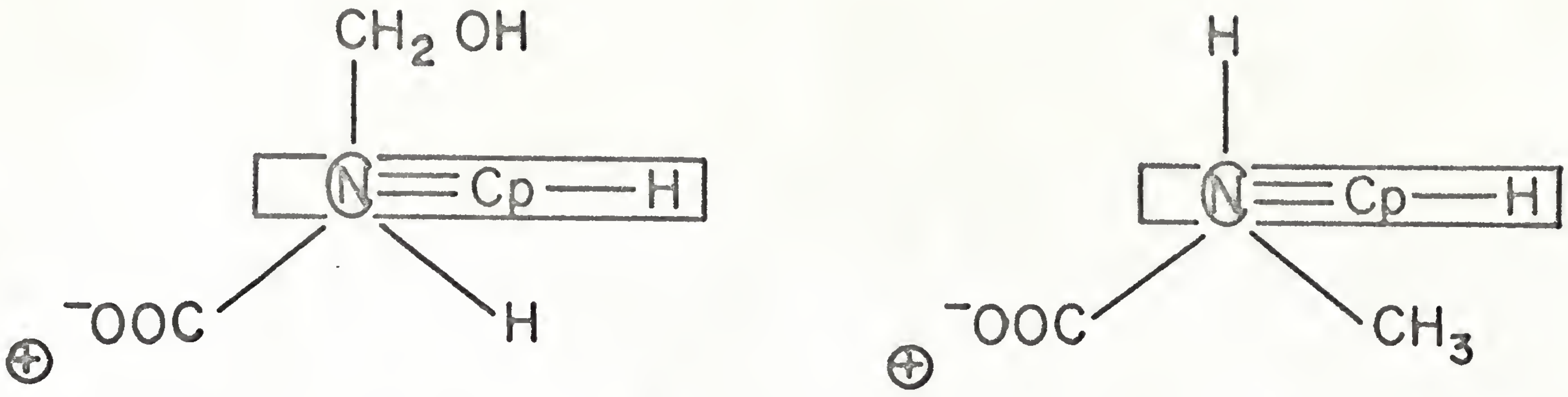


Figure 17. Proposed conformations of pyridoxal-amino acid aldimines oriented for release of the α-hydrogen (I), the α-R group (II) or the α-carboxyl (III) from Dunathan (57). The conventional structural drawings across the top are each reproduced at the bottom as they would be viewed along the axis of the α-carbon to nitrogen bond. In the lower illustrations the substituted pyridine ring is represented by a rectangular box, and the formyl carbon attached to the pyridine ring is labelled C_p.



L - Serine D-Alanine

Figure 18. Possible conformations of L-serine and D-alanine at the active site of L-serine transhydroxymethylase as predicted from the model proposed by Dunathan (57).

group and the α-hydrogen, respectively, in position for cleavage.

This observation was consistent with Dunathan's model; however, a second pertinent point can be made about PLP reaction specificity which was exemplified by the reactions catalyzed by L-serine transhydroxymethylase. The same enzyme could catalyze the cleavage of the methylol group of (+) α-methylserine to a one carbon unit and D-alanine (1), and this reaction, as well as that with the normal substrate, L-serine was reversible. Therefore, the cleavage of the α-hydrogen of D-alanine which preceded its transamination was normal since such a step was required prior to the addition of the methylol group in the reverse of (+) α-methylserine cleavage. Thus the extraordinary feature of the D-alanine transamination by L-serine transhydroxymethylase was that abnormal hydrolysis of the ketimine rather than of the aldimine intermediate occurred, for in the reaction usually catalyzed by this enzyme an amino acid is produced. It is the ability of the enzyme to cleave the α-hydrogen of D-alanine and not to transaminate this amino acid which is significant to and supports the model.

The enzymatically catalyzed α-amino, β-hydroxy acid cleavage reaction is believed to occur with retention of configuration (90). The implication from this is that the bonds which are cleaved and formed during reaction (i.e., an α-methylol and an α-hydrogen sigma bond) are oriented in the same relative position in the active site, just as predicted by the model. These considerations, all consistent with the proposed model, serve to strengthen the argument that, in some instances, the position of a bond in the active site, rather than the nature of the group attached to it, may be more important in determining which bond is cleaved.

A second prediction made by Dunathan was that because the α-carboxylate anion of an amino acid substrate of a decarboxylase would orient itself in the proper geometry for cleavage, these enzymes could show no dual reactivity such as that described above, because the carboxyl group sould always be the only substituent in a position to be labilized.

However, enzymatic decarboxylation had been shown to be reversible (91, 92) and, further, the reaction occurred with retention of configuration about the α-carbon (37) as with the transhydroxymethylase. Therefore, a decarboxylase was capable of cleaving a bond other than the carboxyl but, still consistent with the Dunathan model, both bonds cleaved, the α-carboxyl of the amino acid substrate and α-hydrogen of the amine in the reverse reaction, were oriented in the active site in the same relative position. Note that with the amine substrate, orientation must be assisted by the binding of the side chain to its specific site.

In addition, it was predicted that since the two groups attached to the α-carbon other than the amino and carboxyl were not involved in the decarboxylation reaction, one might expect to see decarboxylation of both isomers of some amino acids in which these other two groups were not structurally too dissimilar. The latter point was supported by the finding of decarboxylation of both D and L-isovaline by α-dialkyl amino acid transaminase.

The prediction that a decarboxylating enzyme would labilize only the α-carboxyl of an amino acid substrate was inconsistent with the finding of the research presented here that α-dialkyl amino acid transaminase labilized the α-hydrogen of L-alanine as well as the α-carboxyl of α-dialkyl amino acids. This problem will be considered after the mechanism of a PLP-dependent racemase, which was inconsistent with the Dunathan model for similar reasons, is discussed.

A racemase which has as its substrates both the D and L form of a given amino acid (93) and which must, then, be capable of cleavage and formation of the α-carbon to hydrogen bond of both isomers was not readily explained. For, if the L amino acid substrate, controlled by the α-carboxyl binding site, bound as in I of Figure 17, with its α-hydrogen in the proper orientation for facile cleavage, then the D isomer must bind with its α-hydrogen in the wrong geometry for cleavage. Dunathan argued that, since the α-hydrogen was properly oriented for cleavage and addition with only one isomer, the racemization reaction would have a high activation energy during the cleavage and formation of the α-hydrogen bond of the other isomer. Thus the rate of a racemization should be slow compared with other PLP -catalyzed reactions.

An alternate proposal to explain the mechanism of a PLP-dependent racemase, embodying the two basic assumptions of Dunathan (which are both reasonable and have precedent) but relaxing some of the restrictions imposed in his model will be presented here and is illustrated in Figure 19. The arguments employed to explain this mechanism will be used to attempt to explain the ability of the α-dialkyl amino acid transaminase to cleave the α-carboxyl of one substrate and the α-hydrogen of another.

First, since the conjugated system of the aldimine is symmetrical about the PLP ring plane, there are two equally "active" orientations for bond cleavage possible, in a given site, i.e., overlapping the pi-electron system above or below the ring plane. This is a point recognized by Dunathan but not utilized to explain any mechanisms.

Second, in the aldimine to ketimine transition of any PLP-catalyzed reaction involving cleavage of one of three possible bonds about the α-carbon, the bond hybridization about the α-carbon shifts from sp^3 to sp^2 with concomitant physical movement of the remaining two bonds to that

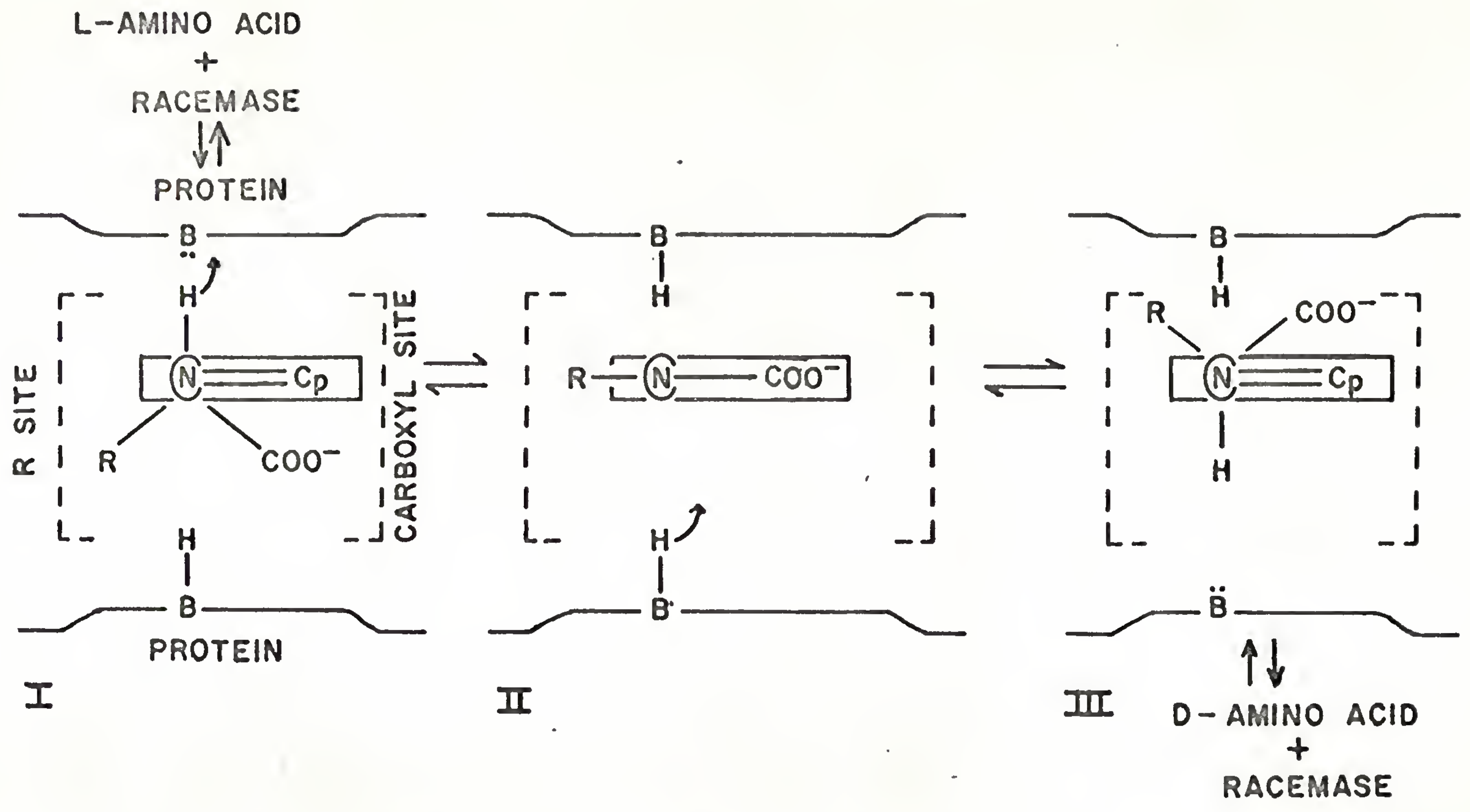


Figure 19. POSSIBLE MECHANISM OF A PLP-DEPENDENT RACEMASE.

carbon into the plane of the conjugated system. Except in decarboxylation, this shift involves the α-carboxyl group, as represented in reaction of structures I to II in Figure 19. Thus there is implied a necessary flexibility of the carboxylate binding site relative to the PLP ring in such enzymes. This could result from a conformational change in the protein structure which accompanied the cleavage step, causing reorientation of a well defined carboxylate binding site relative to the co-factor plane. Such a change in protein conformation would be analogous to the type of changes implied in the "induced fit" hypothesis (51); or it could be imagined that the carboxylate binding site was not shifted but merely was less rigidly defined in space on an inflexible template, such that the carboxyl group was allowed some freedom of movement within its binding site.

If, by either of such possible mechanisms the carboxyl group of a racemase substrate were allowed freedom of movement in the active site over approximately a 90° arc, then the cleavage of protons from either isomer of the substrate in a racemization would require the same energy. This reversible sequence is represented by the steps between structures I and III in Figure 19. It is suggested in this figure that these proton labilizations could be assisted by two properly positioned base-conjugate acid pairs in the protein. This role might be filled by imidazole rings which have been shown to serve as general acid base catalysts of non-enzymatic α-hydrogen transamination with PAL (94). Thus both enantiomers of the DL pair would bind and react equally well assuming that the ionizations of the base-conjugate acids were rapid relative to the formation of aldimines I and III (Figure 19).

It is obvious from the illustrations that the same flexibility argued for the carboxylate binding site must be granted the α-R group binding site as outlined by the dotted lines.

A problem similar to that encountered with racemization arises when one attempts to explain the reaction specificity of the α-dialkyl amino acid transaminase by the Dunathan model. If, as Dunathan proposed, the α-carboxylate anion of each of this enzyme's amino acid substrates binds to the same fixed site on the protein, then the bond which is observed to be cleaved will be oriented for facile cleavage with only one class of substrate (see I and II, Figure 20). If, on the other hand, the α-carboxylate of AIB and the α-hydrogen of L-alanine both must be oriented in the same position for cleavage, then a requirement for carboxylate binding at one specific site can not be satisfied (I and III, Figure 20).

However, the reaction specificity of this enzyme can be reconciled to the Dunathan model as modified to explain

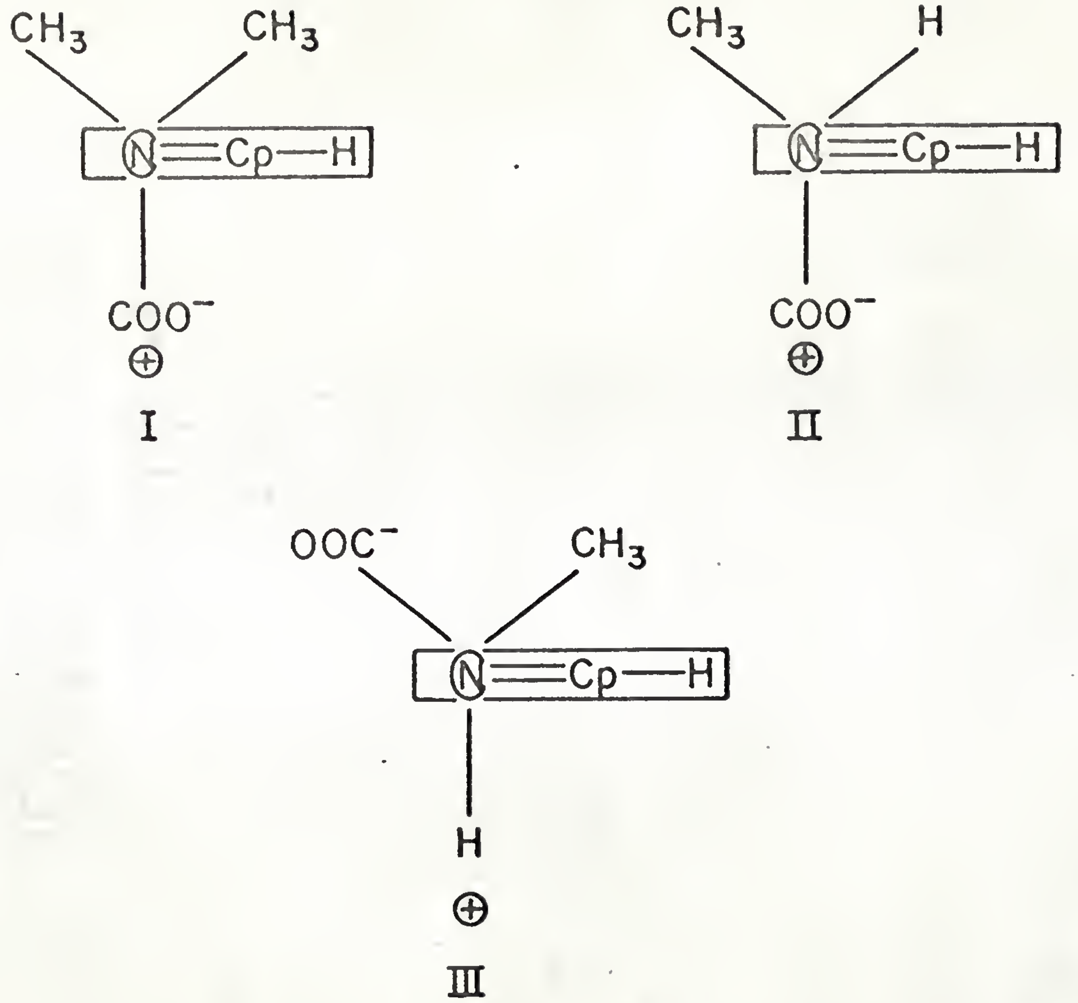


Figure 20. Possible conformations of AIB (I) and L-alanine (II) and (III) in the active site of α-dialkyl amino acid transaminase with the orientation of the α-carboxylate anion of both substrates fixed relative to the plane of the conjugated system (I) and (II) or with the bond to be cleaved fixed relative to this plane (I) and (III).

the racemase mechanism. That is, it may be argued that there is a single, substrate α-carboxylate binding site with the same properties proposed for the racemase and that when the α-carboxylate of any of the amino (or α-keto acid) substrates is properly positioned within the limits of this binding site the bond to the α-carbon which is cleaved or formed will have a geometry perpendicular on one or the other side of the PLP ring plane. Such conformations in the active site of the α-dialkyl amino acid transaminase are illustrated in Figure 21 for both the aldimines and ketimines of AIB (I) and L-alanine (II). The configurational change relative to the ring plane experienced by the bonds to the α-carbon substrate during conversion to sp^2 hybridization in the ketimine is also shown. It can be seen by comparison of the orientation of the ketimine of L-alanine with the aldimine of AIB that both the α-carboxylate and α-R group binding sites must have the same freedom in space suggested in the proposed racemase mechanism.

Orientation exactly perpendicular to the ring plane would represent the lowest energy geometry for the bond which is to be cleaved. Such a geometry can be achieved as well with both substrates even if the carboxyl binding site is given narrower limits, but some freedom of rotation about the α-carbon to nitrogen bond is permitted the PLP

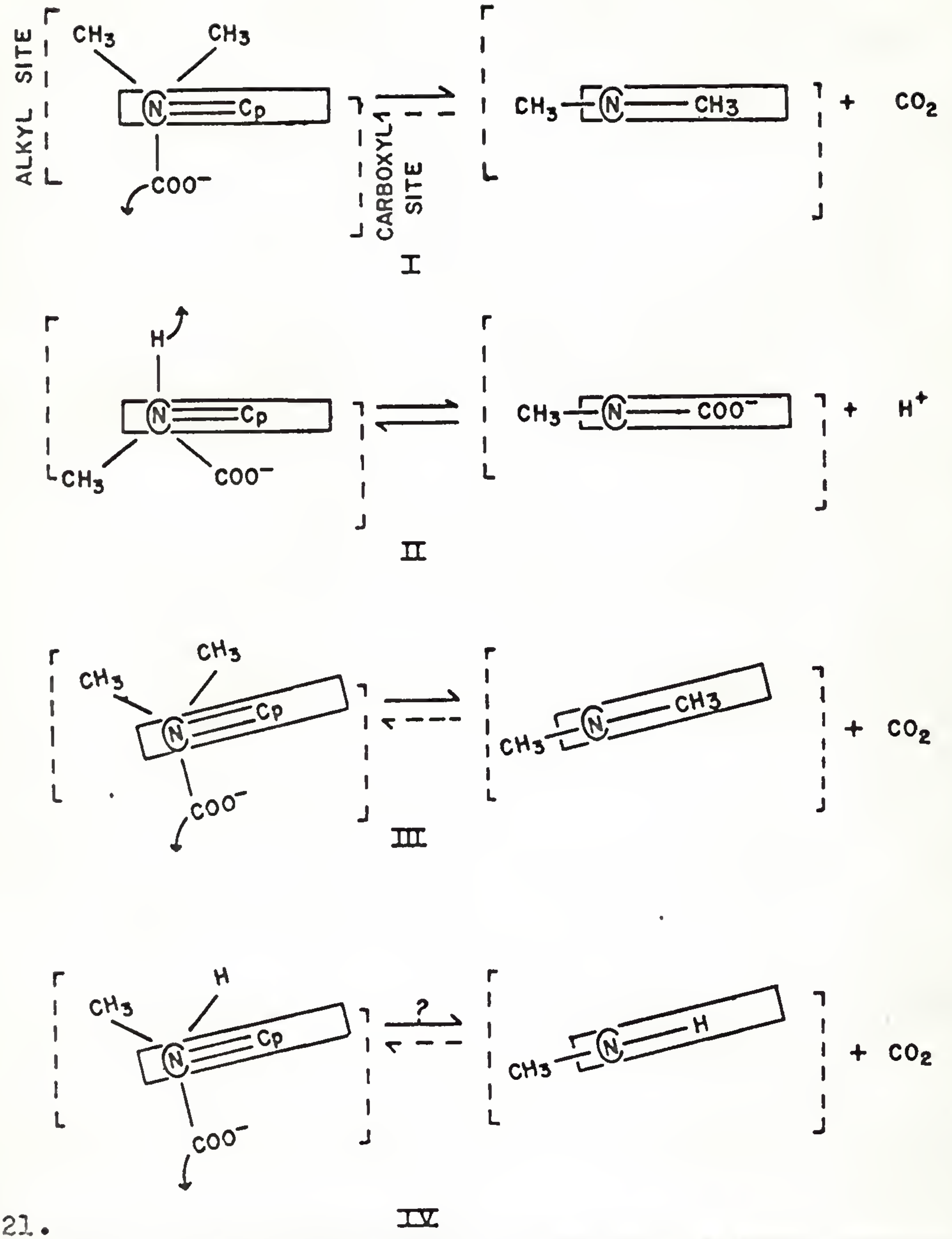


Figure 21.

SOME POSSIBLE CONFORMATIONS OF AIB-AND L-ALANINE-PYRIDOXAL ALDIMINES AT THE ENZYME ACTIVE SITE.

ring in its binding site. This conformation is represented by structures of III, Figure 21, in which the α-carboxyl of AIB in the aldimine complex binds at essentially the same site on the protein as the carboxyl of L-alanine in structures of II of the same figure. Orientation perpendicular to the ring plane to activate the α-carboxyl cleavage is achieved by rotation of the ring plane about the α-carbon to nitrogen single bond relative to the more tightly bound amino acid portion of the complex. This would be accomplished by allowing the same flexibility, or freedom, to co-factor binding as had originally been postulated for the amino acid. In this regard, one is reminded that this enzyme appears to exhibit looser binding of the co-factor than some other PLP enzymes.

As can be seen in drawing IV, Figure 21, L-alanine can assume a conformation in the active site identical to that of AIB without violating any of the restrictions on substrate binding imposed in the description of this model. This conformation has the proper geometry for decarboxylation, yet L-alanine was not decarboxylated at a measurable rate. It can be determined from the results of the molecular orbital calculations of Perault et al. (27) that the ketimine arising from cleavage of the α-hydrogen of an amino acid aldimine with PAL has roughly nine kcal greater delocalization energy than the ketimine formed by α-decarboxylation of the same amino acid. This greater resonance stabilization results from extension of conjugation in the ketimine through the α-carboxylate anion in the first case, but only through the α-carbon in the second. Now, if the geometry of the transition state between the aldimine and the ketimine approaches that of the product ketimine, as is implied by the hypothesis of sigma-pi overlap in this transition state, then the activated states for loss of the α-hydrogen and the α-carboxyl should show similar differences in energy. Therefore, the activation energy for loss of the α-hydrogen will be lower than that for decarboxylation. Because of the exponential relationship of activation energy to the rate constant, a few kcal difference would result in a difference in rates which would prevent detection of decarboxylation of L-alanine by the assay used here.

Finally, this speculative model to explain the reaction specificity of the enzyme must be consistent with the finding that D-alanine was not a good substrate either for decarboxylation-transamination, or for transamination to pyruvate. Assuming that D-alanine was allowed access to the binding site and to form an aldimine with PLP, there are four possible orientations which align either the α -carboxyl or the α-hydrogen of this isomer for cleavage (Figure 22). However, only in structure IV is the α-carboxylate placed within the limits of its proposed binding

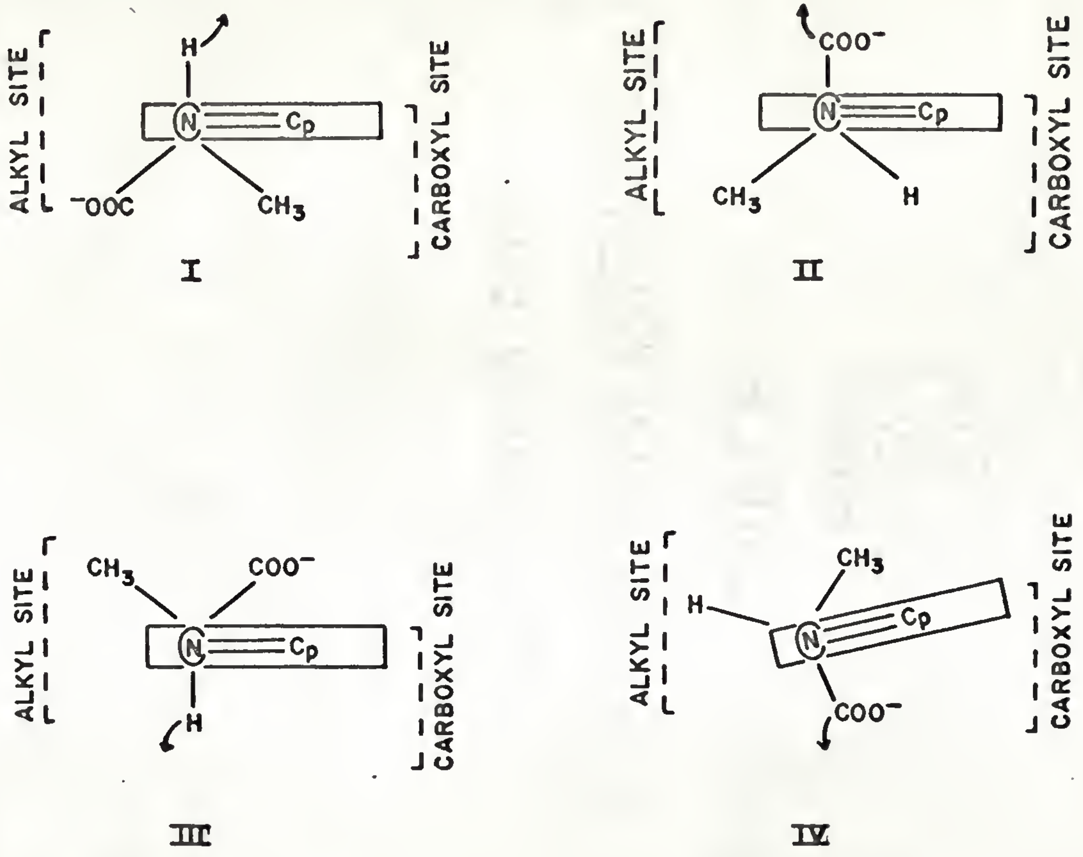


Figure 22. POSSIBLE CONFORMATIONS OF D-ALANINE AT THE ACTIVE SITE.

site. This geometry should allow decarboxylation (in the results of the experiments shown in Table 5 there was some suggestion that D-alanine may have been decarboxylated slowly); but, it is noted that in this orientation there is no alkyl group in an area on the protein where, with all substrates, a methyl or larger alkyl group is positioned. From this observation it must be proposed that, in addition to proper positioning of the α-carboxyl there is also a requirement for an alkyl group at this second site.

If, on the other hand, D-alanine were found not to be an inhibitor of enzyme activity, then the non-reactivity of D-alanine could be due to an alkyl requirement in this site for initial binding of a substrate. It could be that a properly positioned alkyl group, as well as the other essential groups, is necessary to "induce" a conformation of the protein to permit binding and subsequent reaction (51). Snell (95) observed no effect of D-alanine on the spectrum of pyridoxal L-alanine transaminase, while the substrate, L-alanine, destroyed the peak at 415 mμ. This indicated formation of an aldimine with PAL by L- but not D-alanine.

By inhibition and spectral studies of α-dialkyl amino acid transaminase with D-alanine it should be possible to determine the ability of this compound to bind at the active site. Further evidence of a requirement for an alkyl group for binding of the substrate could be gained from measurements of the ability of glycine to serve as a substrate or inhibitor of this enzyme.

SUMMARY

Investigations of the mechanism of bacterial degradation of α-dialkyl amino acids led to the isolation of a single enzyme which transferred the amino group of α-aminoisobutyric acid, 1-aminocyclopentane carboxylic acid, and both enantiomers of DL-isovaline, to pyruvate or closely related aliphatic α-keto acids. The transfer was accompanied by loss of the α-carboxyl group of the amino acid. The products were a ketone from the amino acid substrate, carbon dioxide and the amino acid analog of the α-keto acid.

One hundred seventy-fold purification of the α-dialkyl amino acid transaminase was accomplished by ultracentrifugation of cell sonicates, followed by ammonium sulfate fractionation, gel filtration through Sephadex G-200 and chromatography on DEAE-cellulose.

The molecular weight was estimated from gel filtration data to be 150,000; the observed sedimentation coefficient was 8.3 S.

Enzymatic activity required added co-factor; the apparent K_m for pyridoxal 5'-phosphate (PLP) was 5 $\underline{\mu M}$. The purified preparation contained one mole of co-factor per 400,000 g protein; however, loss of PLP from the protein during purification was suspected.

Carbon dioxide release and butanone production from isovaline were stoichiometric with pyruvate conversion to L-alanine. There was no evidence that decarboxylation of the α-dialkyl amino acids could occur without concomitant transamination.

The final preparation, which appeared 90 - 95 per cent purified upon ultracentrifugation and gel electrophoresis, catalyzed transamination of either L-alanine or L-α-amino-n-butyrate in the presence of added PLP and a suitable α-keto acid. However, with these substrates the amino group transfer was accompanied by cleavage of the α-hydrogen rather than the α-carboxyl of the amino acid.

In addition to the apparent high purity of the enzyme preparation, other findings led to the conclusion that both the α-carboxyl and α-hydrogen labilizing transaminations were catalyzed by the same enzyme. Both activities displayed the same relative specificity for all α-keto acid substrates tested; pyruvate was the most active and α-keto glutarate and probably oxaloacetate were inactive. The α-carboxyl and α-hydrogen labilizing activities chromatographed identically on Sephadex G-200 and DEAE-cellulose and both had the same heat stability. The co-factor requirement during assay was the same for both activities. Transaminase activity for both classes of amino acid substrate was induced in cells grown with either DL-isovaline or DL-α-amino-n-butyrate as nitrogen source.

No significant amount of ^{14}C-carbon dioxide was released from 1-^{14}C-DL-α-amino-_n_-butyrate or either enantiomer of 1-^{14}C-alanine by the purified enzyme. Thus the enzyme exhibited an ability to labilize either an α-hydrogen or an α-carboxyl prior to transamination, with a strong preference for hydrogen cleavage when a properly configured α-carbon possessed both hydrogen and a carboxyl group.

The enzyme did not catalyze detectable exchange of the α-carboxyl of isovaline with ^{14}C-bicarbonate or exchange between isovaline, butanone, and ^{14}C-bicarbonate. Under similar conditions the half reaction involving alanine and pyruvate was fully reversible.

The observed reaction specificity of the α-dialkyl transaminase was discussed in terms of a previous proposal that the reaction specificity of all PLP-dependent enzymes is partially determined by a conformational relationship between substrate and PLP, controlled by substrate α-carboxylate binding in the active site. The reaction specificity of this enzyme was considered to be consistent with the proposed model modified to allow some flexibility of substrate binding sites in the enzyme active site.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Medicine and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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